“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.”

— Albert Einstein, *Relativity: The Special and the General Theory*

Cross-protection studies with swine influenza viruses in pigs and public health aspects

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Thesis for obtaining the degree of Doctor of Philosophy (Ph.D.) in

Veterinary Sciences

2015

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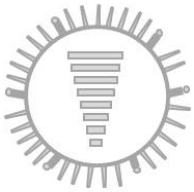
List of Abbreviations

AIV	avian influenza virus
ASC	antibody secreting cells
AUC	area under curve
BALF	bronchoalveolar lavage fluid
CI	confidence interval
CMI	cell-mediated immunity
CTL	cytotoxic t T lymphocyte
DC	dendritic cells
dpi	days post primary inoculation
dpc	days post challenge
EID_{50}	50% egg infectious dose
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-Linked ImmunoSpot
GMT	geometric mean titer
H3N2v	H3N2 variant
HA	hemagglutinin
HAU	hemagglutinating units
HEPA	high-efficiency particulate air-filtered
HI	hemagglutination-inhibition
HRP	horseradish peroxidase-labeled

HPAIV	highly pathogenic avian influenza virus
ID_{50}	50% infection dose
IF	immunofluorescence
IFN	interferon
IHC	immunohistochemical
IL	interleukin
IN	intranasal
ISC	IFN-γ secreting cells
IT	intratracheal
IPMA	immunoperoxidase monolayer assay
LAIV	live attenuated influenza vaccines
M	matrix protein
MDCK	madin-Darby canine kidney
MHC	major histocompatibility complex
ML	maximum-likelihood
MNCs	mononuclear cells
MOI	multiplicity of infection
NA	neuraminidase
NI	neuraminidase-inhibition
NK	natural killer
NP	nucleoprotein

PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH1N1	pandemic H1N1
rH1N1	reassortant H1N1
RNA	ribonucleic acid
RNP	ribonucleoprotein
SCs	secreting cells
SD	standard deviation
SEM	standard error of the mean
SI	swine influenza
SIV	swine influenza virus
TBLN	tracheobronchial lymph nodes
$TCID_{50}$	50% tissue culture infectious dose
Th	T helper
TNF	tumor necrosis factor
TRIG	triple-reassortant internal gene
US	United States
VN	virus-neutralization
WIV	whole inactivated influenza vaccines



Chapter 1

Introduction

1.1 Introduction to influenza viruses

1.1.1 Influenza virus classification

Influenza viruses are the cause of highly contagious acute respiratory disease, known as influenza or “flu”, which has afflicted humans and animals since ancient times. Influenza viruses belong to the *Orthomyxoviridae* family. This family is divided into types A, B, C and D, based on the genetic and antigenic differences between their nucleoprotein (NP) and matrix (M) proteins. Influenza D viruses were first identified in 2011, and so far have only been isolated from pigs and cattle (Collin *et al.*, 2015; Ducatez *et al.*, 2015; Hause *et al.*, 2014; Hause *et al.*, 2013). It is generally assumed that humans are the exclusive natural host for influenza B and C viruses, but both viruses also sporadically infect pigs and some other mammals (Hause *et al.*, 2013; Leyva-Grado *et al.*, 2012; Manuguerra & Hannoun, 1992; Yuanji & Desselberger, 1984). Influenza A viruses infect a wide range of avian and mammalian species, and humans, pigs and horses are the main mammalian hosts (Fouchier *et al.*, 2003; Harder & Vahlenkamp, 2010; Webster *et al.*, 1992). The influenza A viruses are further divided into subtypes, based on the antigenic nature of their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). So far, 18 different HAs (numbered as H1 to 18) and 11 NAs (N1 to 11) have been identified. All known subtypes of influenza A viruses have been found among birds, except subtypes H17N10 and H18N11 that have only been found in bats. Humans and pigs, in contrast, are susceptible to a limited number of subtypes (Table 1). Influenza B, C and D viruses will not be discussed further in this thesis.

Though influenza virus is an important human pathogen, it was first isolated from poultry. A disease causing extremely high mortality in domestic fowl was first described in 1878 and became known as “fowl plague”. As early as 1901 the causative agent was shown to be an ultra-filterable agent, although it was not until 1955 that the close relationship between this agent and mammalian influenza virus was demonstrated. Isolation of influenza virus from pigs also preceded that from humans. The virus was the causative agent of a “novel” disease of pigs, termed “swine influenza (SI)”, with clinical signs similar to those observed in humans. The illness in swine was first described during the 1918 human pandemic. In 1930, Shope demonstrated that swine influenza virus (SIV) could be transmitted between pigs using ultrafiltered material (Shope, 1931), and three years later a similar virus was eventually isolated from a human patient. The virus was propagated by inoculating a

filtrate of the patient's throat washings into the noses of ferrets, which are highly susceptible to influenza virus (Smith *et al.*, 1995). The isolate was later classified as influenza virus and this was followed in 1940 and 1947 by the isolation and classification of influenza B and C viruses, respectively.

The current system of nomenclature of influenza viruses designates the type, host, place, strain number (if any), year of isolation and antigenic subtype of a virus. For example, a swine H3N2 virus isolated in Flanders in 1998 was designated A/Swine/Flanders/1/1998 (H3N2).

1.1.2 Structure of influenza A virus

Influenza A virus has a spherical or filamentous morphology and is medium sized, with a diameter of 80 to 120 nm. The virus is enveloped, and the lipid membrane of the virion is derived from the host cell in which the virus has replicated. The influenza virus genome consists of eight unique segments of negative, single-stranded RNA. Each RNA segment encodes only one protein, except for segment 7 and 8, which encode two. From the surface of the envelope extend the two transmembrane glycoproteins HA and NA (Figure 1). A third transmembrane protein, matrix protein M2, works as ion channel and plays an important role in uncoating of the virus in the cellular endosome. The matrix protein M1 forms a layer beneath the envelope and thus gives structure to the virus and encapsidates the ribonucleoprotein (RNP) complexes. RNP complexes consist of ribonucleic acid (RNA) associated with NP as well as the polymerases PA, PB1 and PB2 that are responsible for RNA replication and transcription. The proteins NS1 and NS2 are also associated with the virus and are no longer considered to be non-structural proteins: NS2 is found in the virion and mediates the export of RNPs from the nucleus to the cytoplasm (O'Neill *et al.*, 1998), while NS1 is found mainly in infected cells but also at low levels within the influenza virion. It was shown to repress innate antiviral mechanisms in infected host cells (Hutchinson *et al.*, 2014). In some strains, the RNA segment 2 encodes not only the PB1 protein, but also a second protein, called PB1-F2.

1.1.3 Emergence of novel influenza viruses and virus evolution

Due to the lack of proofreading of RNA polymerase, mutations in the influenza virus genome occur frequently, resulting in an error rate of around 1×10^{-5} substitutions per nucleotide per replication cycle (Parvin *et al.*, 1986; Sanjuan *et al.*, 2010). The

surface glycoproteins, HA and NA, are the most important antigens for inducing protective immunity in the host. Due to an accumulation of mutations in the HA and NA genes, the proteins will undergo minor structural changes over time, which are known as "**antigenic drift**". Antigenic drift allows influenza viruses to escape from pre-existing immunity, and it is the reason why the virus strains in the human influenza vaccines are updated every few years.

Due to the segmented genome, influenza viruses can "mix and match" gene segments upon simultaneous infection of the same host cell with two or more different viruses. This is designated "**genetic reassortment**". In some cases, a new reassortant virus occurs with a different combination of HA and/or NA genes from the virus that was previously dominant in the population. This is known as "**antigenic shift**". While antigenic drift is the cause of the regular recurrence of influenza epidemics, antigenic shift may cause an **influenza pandemic**, which refers to an epidemic that spreads on a worldwide scale and infects a large proportion of the human population. The pandemic occurs when a virus meets two requirements: 1) a large proportion of the population lacks immunity against the virus, and 2) the virus has the ability to spread efficiently from person to person. Three different mechanisms may lead to the emerge of a pandemic influenza virus (Figure 2): 1) Direct transfer of whole virus from another species. This is probably what occurred in 1918 when the H1N1 "Spanish flu" virus entered the human population from the avian reservoir. It resulted in the most devastating pandemic in known history and was responsible for an estimated 20 to 40 million deaths (Patterson & Pyle, 1991). 2) Genetic reassortment between animal and human influenza viruses,

Table 1. Influenza A viruses HA and NA subtypes that have become established in birds, humans, pigs and other animals (adapted from the US CDC website http://www.cdc.gov/flu/about/viruses/transmission.htm#subtypes)

HA					NA				
Subtype	Birds	Human	Pigs	Bats / Other	Subtype	Birds	Human	Pigs	Bats / Other
H1					N1				
H2					N2				Dog
H3				Dog, horse	N3				Seal
H4					N4				
H5					N5				
H6					N6				
H7				Horse	N7				Horse
H8					N8				Dog, horse
H9					N9				
H10					N10				
H11					N11				
H12									
H13									
H14									
H15									
H16									
H17									
H18									

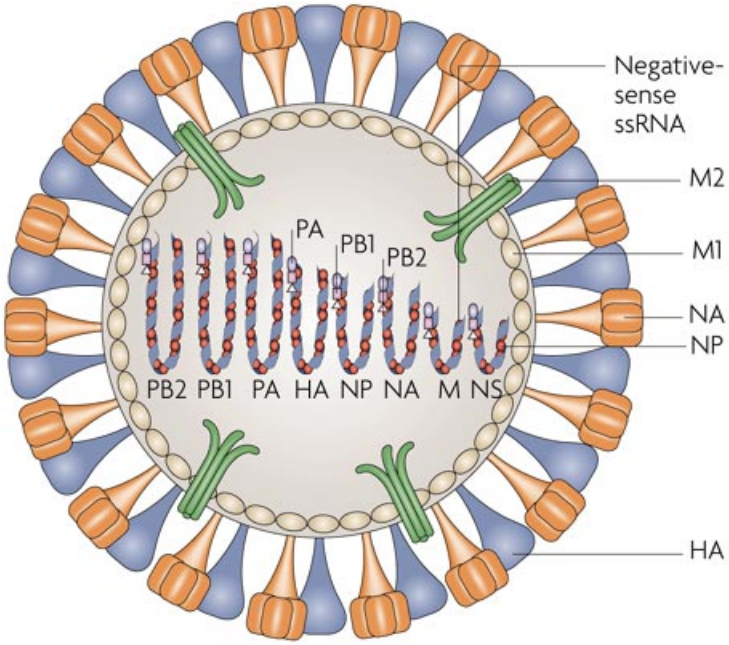


Figure 1. The structure of influenza A virus (adapted from Nelson and Holmes 2007).

producing a virus with a new HA subtype. As a result of reassortment, the H2N2 "Asian flu" virus, responsible for the pandemic of 1957, acquired the HA, NA and PB1 gene segments from an avian virus and kept the other five segments from the human H1N1 strain already in circulation. The H3N2 "Hong Kong flu" virus of 1968 acquired HA and PB1 segments of avian origin and the remaining six segments from the H2N2 virus. 3) Recycling of an HA that has disappeared from the human population long ago. The 1918 H1N1 pandemic influenza virus is the common ancestor of human seasonal and "classical" swine H1N1 influenza viruses, and has undergone significant antigenic drift in humans, but nearly remained in antigenic stasis in swine (Kash *et al.*, 2010; Skountzou *et al.*, 2010). The virus that caused the 2009 H1N1 pandemic is a reassortant virus that contains the NA and M genes from the European H1avN1 SIV lineage, and the remaining genes from the North American triple-reassortant H1N2 SIV lineage, which bears the "classical" swine H1 (Garten *et al.*, 2009; Smith *et al.*, 2009). Consequently, only people born before 1940-50 had been previously exposed to human seasonal H1N1 viruses with an H1 related to that of 2009 pandemic H1N1 virus (pH1N1), and a pandemic was possible because younger people lacked cross-reactive anti-H1 antibodies (CDC, 2009; Hancock *et al.*, 2009; Ikonen *et al.*, 2010; Skowronski *et al.*, 2011).

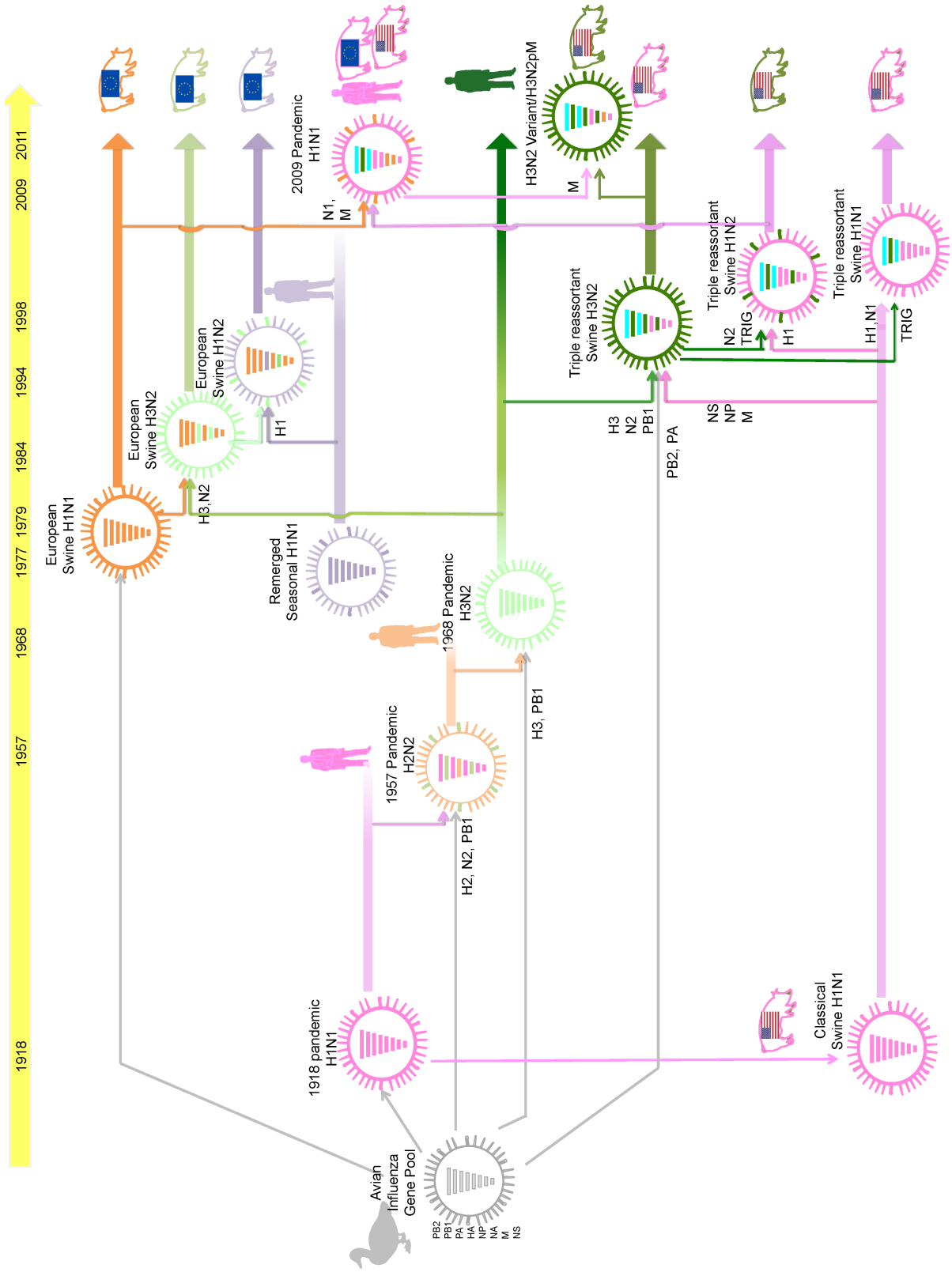


Figure 2. Genetic relationships among influenza A viruses of human, European swine, and (selected) North American swine lineages, 1918-2009.

1.2 Influenza A viruses in pigs and in humans

1.2.1 Epidemiology of swine influenza viruses worldwide

Three influenza virus subtypes – H1N1, H3N2 and H1N2 – are currently circulating in swine worldwide, but the origins and the antigenic and genetic characteristics of these SIV subtypes differ in different continents or regions of the world. In Europe, the predominant H1N1 SIVs are entirely of avian origin. They were introduced from waterfowl to pigs in 1979 and are referred to as the avian-like H1N1 (H1avN1) SIV (Pensaert *et al.*, 1981). Shortly after the human Hong Kong influenza pandemic in 1968, H3N2 influenza viruses were isolated in European pigs (Ottis *et al.*, 1982; Tumova *et al.*, 1980), but they only became widespread after reassortment with the H1avN1 SIV in the early 1980s . Those reassortant H3N2 SIVs inherited the 6 internal genes from the latter lineage, and retained the human-like HA and HA genes (Campitelli *et al.*, 1997; Castrucci *et al.*, 1993). Another reassortant virus of H1N2 subtype was first reported in 1994 in the United Kingdom and then throughout Europe (Brown *et al.*, 1995; Brown *et al.*, 1998; Van Reeth *et al.*, 2000). This human-like H1N2 (H1huN2) virus contains an HA of a human seasonal H1N1 virus from the 1980s, a NA of swine H3N2 virus origin and internal genes of swine H1avN1 virus origin (Brown *et al.*, 1998). Thus, the three major virus lineages share common internal genes, but they have genetically and antigenically distinguishable HAs and/or NAs.

These three major SIV lineages have been co-circulating for many years in the European swine population, but the prevalence and incidence of individual subtypes varied from one country to another (Kyriakis *et al.*, 2011; Kyriakis *et al.*, 2013; Van Reeth *et al.*, 2008). Since 2000, new reassortant viruses between these three SIV lineages or between SIVs and human seasonal influenza viruses have been detected occasionally (Kyriakis *et al.*, 2011), but the situation was rather stable in European pig herds until the emergence of the swine-origin pH1N1 virus in 2009 (Simon *et al.*, 2014b). Approximately one month after the isolation of the pH1N1 virus in humans, the virus was transmitted back to swine, with the first pH1N1 virus being isolated from a pig herd in Alberta, Canada, in May 2009 (Howden *et al.*, 2009). Since then, pH1N1 viruses were associated with outbreaks in swine in Europe (Howard *et al.*, 2011), Asia (Vijaykrishna *et al.*, 2010), Africa (Njabo *et al.*, 2012), Australia (Holyoake *et al.*, 2011), and South America (Pereda *et al.*, 2010). Pigs were shown to be highly susceptible to this pH1N1 virus (Brookes *et al.*, 2010; Lange *et al.*, 2009), and the

establishment of pH1N1 in pigs has complicated the SI epidemiology in many countries because the virus has reassorted with pre-existing H1N1, H1N2 and H3N2 SIVs. In Europe, a growing number of reassortants between the four SIV lineages has been reported in recent years (Harder *et al.*, 2013; Moreno *et al.*, 2013; Rose *et al.*, 2013; Trebbien *et al.*, 2013), and viruses containing 1 to 7 genes of the pH1N1 virus with the HA and/or NA of previously established SIVs are now common in several European countries (Chiapponi *et al.*, 2014; Lange *et al.*, 2013; Simon *et al.*, 2014a).

In North America, SI was first recognized in pigs in the Midwestern United States (US) in 1918 as a respiratory disease that coincided with the human Spanish flu pandemic. Since then, this classical swine lineage H1N1 virus derived from the 1918 pandemic virus established itself in US swine and has remained relatively stable at the genetic and antigenic levels for nearly 80 years. The epidemiology of SIVs in the US dramatically changed after 1998 when triple-reassortant H3N2 viruses containing gene segments from the classical swine virus (NP, M, NS), H3N2 human seasonal virus (PB1, HA, NA) and avian influenza virus (AIV) (PB2, PA) (Zhou *et al.*, 1999) became successfully established in the pig population (Webby *et al.*, 2000). This genome composition of SIV is referred to as the triple-reassortant internal gene (TRIG) cassette (Vincent *et al.*, 2008b). These triple-reassortant H3N2 viruses cocirculated with classical H1N1 viruses in swine and exchanged genome segments via reassortment, generating reassortant H1N1 or H1N2 viruses (Karasin *et al.*, 2002; Webby *et al.*, 2004). The triple-reassortant H3N2 SIVs were classified as H3 clusters I to III, a result of three separate human seasonal H3 gene introductions in 1995 (H3-I), 1997 (H3-II), and 1996 (H3-III), respectively (Richt *et al.*, 2003). Swine H3-IV evolved from cluster III and was first reported in 2005, but remained relatively stable until 2010 (Olsen *et al.*, 2006). From 1998 to 2009, the majority, if not all H1 and H3 viruses isolated from swine contain the TRIG constellation (Evseenko *et al.*, 2011; Lorusso *et al.*, 2011; Nfon *et al.*, 2011a; Nfon *et al.*, 2011b).

Currently, viruses of six distinct antigenic HA types are cocirculating in swine in the US and Canada: H1α, H1β, H1δ, H1γ, pandemic H1, and H3 (cluster IV) (reviewed in (Lorusso *et al.*, 2012)). The introduction of the pH1N1 virus and reassortment with triple-reassortant H3N2 SIVs has altered both the genotype and phenotype of the previously stable swine H3-IV viruses. At least 10 H3N2 genotype patterns emerged in the US (Kitikoon *et al.*, 2013). The H3N2 genotype that inherited only the M gene

from the pH1N1 virus in particular has raised public health concerns in recent years. This virus, called “H3N2pM” when isolated from pigs, and called “H3N2 variant” or “H3N2v” when isolated from humans, was detected more frequently than any other genotype (~36 % of H3N2-TRIG/pH1N1 reassortant viruses) (Kitikoon *et al.*, 2013).

In Asia, the epidemiology of SIVs is far more complicated than that in Europe or North America. The first wholly human-like H3N2 were first isolated from pigs in Taiwan soon after the Hong Kong pandemic (Kundin, 1970). In the next years, almost all of the major human H3N2 variants could be introduced into the pig population, but most were prevalent at a low level within a small geographic location, and failed to become established in pigs except for the A/Port Chalmers/1/73, A/Victoria/3/75 and A/Sydney/05/97 variants (Shortridge *et al.*, 1979; Shortridge & Webster, 1979; Shortridge *et al.*, 1977; Yu *et al.*, 2008a). Besides those entire human-like viruses, various H3N2 reassortant viruses have been reported throughout Asia, and some resemble European double reassortant or North American triple reassortant virus lineage (Pan *et al.*, 2009; Takemae *et al.*, 2008; Vijaykrishna *et al.*, 2011; Yu *et al.*, 2008a), whereas other SIVs have emerged locally and are clearly unique for Asia (Nerome *et al.*, 1995; Ngo *et al.*, 2012; Shu *et al.*, 1994; Sun *et al.*, 2009). The cocirculating of so many genetically diverse SIVs has further resulted in multiple and complex reassortant viruses (Bi *et al.*, 2010; Chen *et al.*, 2014; Yu *et al.*, 2008a).

In South America, limited serological surveys in Venezuela, Colombia and Brazil have revealed that H3N2 subtypes are prevailing in swine (Perfumo, 2010). However, neither virus isolation nor sequencing analysis had been reported before 2008. In late 2008, a wholly human H3N2 was isolated from pigs, which shares of 96-98% nucleotide identities with human seasonal H3N2 viruses from 2000-03 (Cappuccio *et al.*, 2011). This represents an independent human-to-swine transmission event that is distinguishable from those in Europe and North America.

1.2.2 Antigenic evolution of H3N2 viruses in humans and in pigs

Most, if not all H3N2 SIVs contain human-origin HA and NA genes, but the antigenic evolution of H3N2 viruses is usually slower in pigs than in humans (Van Reeth *et al.*, 2012). Because of the short average life span of pigs, SIVs evolution may be determined to only a limited extent by herd immune pressure, which is the driving force of antigenic drift of influenza viruses in humans. Whereas the human H3N2

virus requires frequent antigenic changes of HA to ensure that a sufficiently large pool of immunologically susceptible hosts is available, the vast majority of pigs are killed at the age of 6 months, and the susceptible pig population is continuously renewed, thus limiting immune pressure. Only adult sows used for breeding live long enough to experience more than one influenza season, and therefore may create some degree of immune pressure, leading to the (slow) antigenic drift of SIVs (de Jong *et al.*, 2007).

It has been shown that European swine and human seasonal H3N2 viruses have a similar rate of genetic evolution in their HA1 region during 1982-2002 (~5 × 10^{-3} and ~6 × 10^{-3} substitutions per nucleotide per year, respectively), but swine viruses apparently mutate in regions that are less important for the antigenic properties (de Jong *et al.*, 2007; Nerome *et al.*, 1995). Moreover, antigenic drift in the HA1 is ~6 times slower in European H3N2 SIVs than in their human counterparts (de Jong *et al.*, 2007). As for human seasonal H3N2 viruses, 13 different antigenic clusters have been identified between 1975 and 2011 (Smith *et al.*, 2004; Westgeest *et al.*, 2014) and the human H3N2 vaccine component has been updated 21 times during this period. European H3N2 SIVs have been grouped into two antigenic clusters (de Jong *et al.*, 2007). Compared to cluster 1 viruses (1984-1993), the later cluster 2 viruses (1993-2001) showed reduced cross-reactivity with A/Port Chalmers/1/73, which is assumed to be the ancestor human virus for European H3N2 SIVs, in hemagglutination-inhibition (HI) assays with post-infection ferret sera (de Jong *et al.*, 2007; de Jong *et al.*, 1999). Yet sera from pigs vaccinated with a commercial A/Port Chalmers/1/73 vaccine discriminated less well between both antigenic clusters than the ferret sera, and the A/Port Chalmers/1/73-based vaccine significantly reduced lung virus titers against challenge with the cluster 2 viruses sw/Flanders/1/98, sw/Oedenrode/7C/96 and sw/Gent/172/08 (De Vleeschauwer *et al.*, 2015; Heinen *et al.*, 2001b; Van Reeth *et al.*, 2001).

The North American H3N2 SIVs have shown more antigenic diversity than their European counterparts. Antigenic diversity has been demonstrated between the four clusters of H3N2 SIVs (Gramer *et al.*, 2007; Richt *et al.*, 2003). The newly emerged genotypes within cluster IV viruses since 2011 show an increasing antigenic diversity. Antisera produced against contemporary swine H3 isolates had low cross-HI reactivity to cluster I virus, older cluster IV isolates and inconsistent cross-reactivity among the new subclusters (Kitikoon *et al.*, 2013). Based on the cross-HI assays

with post-vaccination swine sera, cluster IV viruses circulating between 2006 and 2013 have been differentiated into two major antigenic groups, with some isolates from 2010-2013 located as outliers in the antigenic map (Lewis *et al.*, 2014). Genetic analysis has identified 6 amino acid positions (145, 155, 156, 158, 159, and 189) in HA1 that are critical for the antigenic phenotype of those H3N2 SIVs, and changes in only one or two of these amino acids are thought to be sufficient for antigenic switches. The key amino acid positions are strikingly similar to the 7 amino acids that have been shown to drive human antigenic switches from 1968 to 2003 (Koel *et al.*, 2013).

As most studies focus on the antigenic drifts of the HA, knowledge on the antigenic evolution of the NA is relatively limited. For humans seasonal H3N2 viruses during 1968-2009, the overall rates of evolutionary change were lower for NA than for HA1 at the nucleotide level (Westgeest *et al.*, 2012). Neuraminidase-inhibition assays with ferret post-infection sera has demonstrated the NA drifted antigenically in a noncontinuous pattern, which did not correspond closely with HA drift (Sandbulte et al., 2011). So far, knowledge about the genetic and antigenic drift of NAs in H3N2 SIVs remains quite limited.

1.2.3 Transmission of H3N2 influenza viruses from swine to humans

Some influenza viruses may remain in evolutionary stasis in pigs for decades after the disappearance of their ancestor from the human population. Those SIVs may jump back into humans when the human population immunity has waned to sufficiently low levels, and the pH1N1 is the most notable example. For H3N2 SIVs, only sporadic and dead-end human infections have been reported occasionally since the 1990s (Figure 3). Due to weak or non-existent surveillance systems in large parts of the world, estimates of the prevalence of these zoonotic infections should be made with caution.

In 1993, the European H3N2 SIV was isolated from a one-year-old girl and a two-year-old boy in the Netherlands (Claas *et al.*, 1994). The children, who were living in geographically distinct regions, developed mild respiratory symptoms. Neither child had been in close contact with pigs, but the father of the boy had regular contact with pigs through his occupation. A similar H3N2 SIV was isolated from a 10-month-old girl in Hong Kong in 1999 (Gregory *et al.*, 2001). In 2014, a H3N2 SIV infection was isolated from a 67-year-old immunocompromised man, and the local pig farm that he

visited before the sickness was identified as the source of infection (Piralla *et al.*, 2015).

The first reported North American triple-reassortant H3N2 SIV zoonotic infection case occurred in Ontario, Canada, in 2005. The virus was isolated from an adult swine farmer (Olsen *et al.*, 2006). In 2006, another case of H3N2 SIV infection was identified in a Canadian 7-month-old boy. The infant lived on a farm with pigs and other animals but he had no direct contact with those animals (Robinson *et al.*, 2007). Between 2009 and 2010, triple-reassortant H3N2 SIVs were detected in 7 people in the US ranging from 9-month-old to 45-year-old (Shu *et al.*, 2012). From July 2011 to October 2014, a total of 343 cases of infection with a novel swine-origin H3N2v virus were reported in 13 states in the US, with 18 people being hospitalized and 1 death (http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm). Of the eight gene segments of H3N2v, all but the M gene were similar to those of triple-reassortant H3N2 SIV currently circulating in North America, and to those of H3N2 SIVs isolated from humans during 2009-10. However, the M gene of H3N2v virus is inherited from the pH1N1 virus. The patients have a median age of 7 years old, and the majority reported agricultural fair attendance (93%) and/or contact with swine (95%) prior to illness. Limited person-to-person H3N2v virus transmission was reported, but there was no evidence of sustained person-to-person transmission (Jhung *et al.*, 2013).

1.3 Pathogenesis and immune responses to influenza A virus in the pig

1.3.1 Clinical features and pathogenesis of influenza viruses in the pig

Swine influenza is an acute febrile, respiratory disease characterized by fever (usually in the range 40.5-41.7 ºC), apathy, anorexia, coughing. The disease may progress to open-mouthed breathing and dyspnea, especially when animals are forced to move. Generally, recovery is rapid, usually 5-7 days after the onset of clinical signs. Uncomplicated SIV infections cause low mortality (usually less than 1%), but morbidity can reach 100% (Van Reeth *et al.*, 2012). Secondary bacterial infections can increase the severity of illness. The gross lesions found in uncomplicated SI are mainly those of a viral pneumonia and are most often limited to the apical and cardiac lobes of the lungs, although in severe cases more than half of the lung may be affected. The altered lung areas are consolidated and dark red or purple-red in color, contrasting sharply with normal tissue.

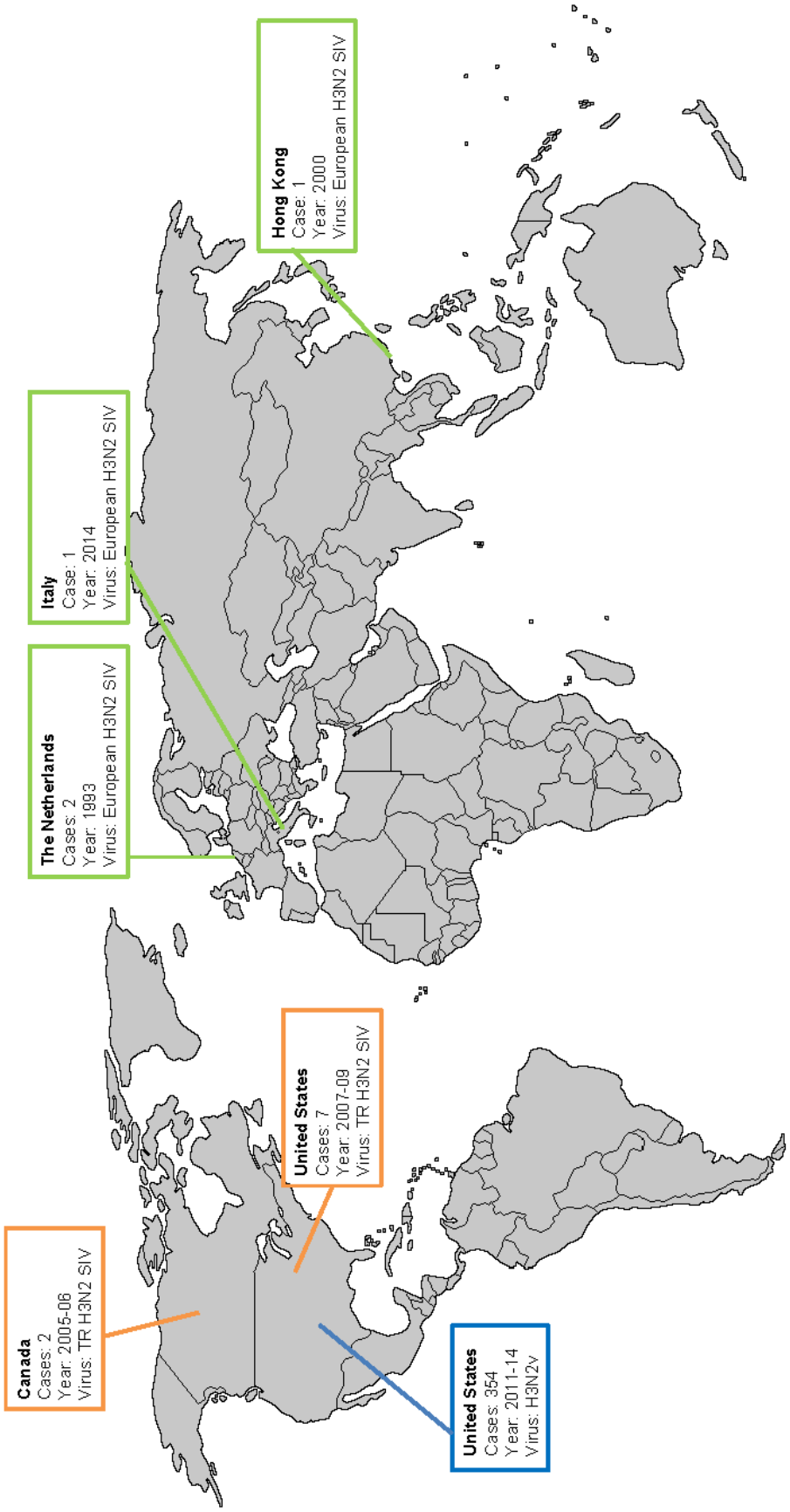
Infection with SIV is generally limited to the epithelial cells of nasal mucosa, tonsils, trachea, bronchi, bronchioles and alveoli (Brown *et al.*, 1993; De Vleeschauwer *et al.*, 2009a; Heinen *et al.*, 2000; Lanza *et al.*, 1992). Besides those tissues, SIVs can also be isolated from ethmoid, tracheobronchial lymph nodes (TBLN) and bronchoalveolar lavage fluid (BALF). Virus shedding and transmission occur almost exclusively via the respiratory route, and the brain stem is the single extra-respiratory tissue where low titers of virus were occasionally detected by both polymerase chain reaction (PCR) assay and virus isolation, but no specific infected cells were detected by immunofluorescence (IF) assay (De Vleeschauwer *et al.*, 2009a). Only one study reported viremia of low titer and short duration (Brown *et al.*, 1993).

Infection and replication of influenza viruses in host cells progresses very rapidly, while virus clearance is also extremely rapid. In most experimental studies, nasal virus shedding starts at 1 day post-inoculation (dpi) for 5-6 days, occasionally 7 days. SIVs have a preference for the lower versus upper respiratory tract. Virus titers in the lungs may reach up to 10^8 egg infectious doses 50 (EID_{50}) per gram of tissue (Haesebrouck & Pensaert, 1986; Van Reeth *et al.*, 1998), and IF and immunohistochemical (IHC) studies demonstrate massive numbers of viral antigen-positive cells in the bronchial, bronchiolar, and alveolar epithelia, compared to fewer positive cells in the nasal mucosa (Brown *et al.*, 1993; De Vleeschauwer *et al.*, 2009a; Haesebrouck & Pensaert, 1986; Van Reeth *et al.*, 1998).

Infection with SIV can be easily reproduced by experimental inoculation of influenza naïve pigs using aerosol, intranasal (IN), or intratracheal (IT) inoculation routes. However, the kinetics of virus replication in the respiratory tract, clinical manifestations and the severity of lung inflammation vary remarkably depending on the inoculation route and dose. Typical SI clinical signs, that is, high fever (≥40.5 °C), lethargy, anorexia, pronounced tachypnea and expiratory dyspnea, and lung inflammation is most reproducibly accomplished by IT inoculation of high doses of virus (≥$10^{7.0}$ EID_{50}) (De Vleeschauwer *et al.*, 2009a; Van Reeth *et al.*, 1998; Van Reeth *et al.*, 2002). Less invasive methods, such as IN inoculation or IT inoculation with a low dose, result in mild or entirely subclinical infections (Landolt *et al.*, 2003; Larsen *et al.*, 2000).

Proinflammatory cytokines produced by the host during the very acute stage of an infection probably play a critical role in SI disease development. Titers of interferon-α and -γ (IFN-α and -γ), tumor necrosis factor-α (TNF-α), interleukin-1, -6 and -12 in

BALF are extremely high after high-dose IT inoculation, and they coincide with the peak of disease (Van Reeth *et al.*, 1998; Van Reeth *et al.*, 2002). Experimental studies support the concept that a high amount of virus that reaches the deeper airways and the resulting production of infectious virus likely determine the extent of cytokine production in the lungs, which in turn determines the severity of illness. However, many cytokines also have antiviral and immunostimulating effects, and thus may contribute to the clearance of influenza viruses. Little differences have been found in the pathogenesis or virulence between SIV lineages or strains (Van Reeth *et al.*, 2012). The pH1N1 virus has shown a similar pathogenetic course in pigs as the SIVs that are endemic in swine populations (Brookes *et al.*, 2010; Lange *et al.*, 2009). Inoculation of pigs with AIV or some human influenza virus strains may result in a mild to subclinical infection, consistent with low-to-moderate virus titers in the respiratory tissues (De Vleeschauwer *et al.*, 2009b; Landolt *et al.*, 2003; Van Reeth *et al.*, 1998).



Abbreviations: SIV, swine influenza virus; TR, triple-reassortant.

Figure 3. Human infections with H3N2 swine influenza viruses worldwide.

1.3.2 Immune responses to influenza viruses in the pig

Much of our knowledge about immune mechanisms operating against influenza virus has been gained from studies in humans and rodents, there are only a limited number of specific studies in swine. In general, after exposure to an influenza virus, the first line of host defense is formed by the innate immune system, which is quick but lacks specificity and memory. It consists of physical barriers (i.e., mucus and collectins) and innate cellular immune responses. Innate immune cells like macrophages, dendritic cells (DC) rapidly exit the respiratory tract, and travel via afferent lymph to regional lymph nodes or via the blood to the spleen, to deliver antigen to naïve and memory T and B cells. Alveolar macrophages and natural killer (NK) cells also have a direct role in limiting virus replication and dissemination. Interferons (IFN) are produced early in infection and may reduce viral spread by inducing an antiviral state in host cells and by activation of cytotoxic T cells (CTLs) and NK cells, thereby contributing to recovery from infection (van de Sandt *et al.*, 2012).

In addition to the non-specific immune mechanisms, adaptive immune responses are needed for the ultimate elimination of the virus. The latter is highly specific but is relatively slow upon first encounter with a pathogen. However, as a result of immunological memory, the adaptive response reacts faster and stronger after a second encounter with the same or a closely related pathogen. The adaptive immune response consists of cellular (virus-specific $CD4^+$ and $CD8^+$ T cells) and humoral (virus-specific antibodies) immunity.

The cell-mediated immunity (CMI), mediated by virus-specific $CD4^+$ T cells and $CD8^+$ T cells, is directed against conserved regions in all internal and surface proteins of the virus. To be recognized by T cells, viral antigens must be degraded into small immunogenic peptides in antigen-presenting cells and presented on the surface of these cells in association with molecules of the major histocompatibility complex (MHC). $CD4^+$ T cells are activated by peptides derived from exogenous, phagocytosed antigens, which are displayed by MHC class II molecules. In contrast, $CD8^+$ T cells are activated by peptides that are generally derived from de novo, intracellular synthesis of viral protein and these are presented in association with MHC class I molecules. Activated $CD4^+$ T cells can be differentiated into $CD4^+$ T helper 1 (Th1) or Th2 cells (Zhu & Paul, 2010). Th2 cells stimulate B cells to proliferate and differentiate into antibody producing plasma cells (Okoye & Wilson,

2011). Th1 cells are mainly involved in helping $CD8^+$ T cells to proliferate and differentiate to CTLs (Mosmann *et al.*, 1986), and are essential for the induction of memory $CD8^+$ T cells (Deliyannis *et al.*, 2002). The CTLs are considered to be the key players of the CMI response. After activation, they migrate to the site of infection where they recognize and kill influenza virus-infected cells and the viruses reproducing within them (Nakanishi *et al.*, 2009). CTLs are mainly directed against epitopes on the NP, which is highly conserved among influenza A viruses.

Neutralizing antibodies are the most important line of defense against influenza infection, and they are mainly directed against the viral HA protein. By binding to the HA receptor binding site in the globular head, neutralizing antibodies can block viral entry by either preventing the attachment of the virus to sialic acid receptors on host cells or by interfering with HA-mediated viral fusion (Brandenburg *et al.*, 2013). The NA protein is also a target of neutralizing antibodies. By inhibiting the enzymatic activity of NA, NA antibodies can prevent influenza viruses from penetrating host mucus layer that is rich in heavily sialylated glycoproteins, and therefore prevent influenza viruses from entering respiratory epithelia (Cohen *et al.*, 2013), and limit the fusion of the influenza virus into host cells (Su *et al.*, 2009). Those antibodies also interfere with the last phase of the viral replication cycle by limiting newly formed viruses releasing from host cells. Antibodies to the more conserved NP and M proteins are also produced during influenza virus infection (Potter & Oxford, 1979). Though they cannot prevent an infection, they contribute to the protection by killing infected cells through antibody-dependent mechanisms.

Experimental pig infection studies with SIV have shown that antibodies can be detected in serum by HI and virus-neutralization (VN) assays at 7-10 days post-infection and peak by 2-3 weeks (Larsen *et al.*, 2000; Van Reeth *et al.*, 2006). Antibodies to the NA and NP are detected in post-infection swine sera (Heinen *et al.*, 2001a; Heinen *et al.*, 2000; Van Reeth *et al.*, 2003), as well as moderate antibody levels to M1, and low and variable antibody levels to the external part of M2 (Kitikoon *et al.*, 2008). Antibodies to NP have also been detected in nasal secretions and BALF (Heinen *et al.*, 2001a; Heinen *et al.*, 2000; Kitikoon *et al.*, 2006; Kitikoon *et al.*, 2009; Larsen *et al.*, 2001; Larsen *et al.*, 2000). The HA-specific IgA and IgG responses have been detected in respiratory secretions and BALF, as well as in serum, where IgM is the first immunoglobulin produced (Lee *et al.*, 1995). Both IgA and IgG antibodies can be locally produced in the respiratory tract, and the SIV-specific

antibody-secreting cell responses peaked at 2-3 weeks post-infection (Larsen *et al.*, 2000). IgG antibodies can also transudate from serum into alveolar epithelium and contribute to the protection in the lung. Data on CMI in the pig are even more limited, and studies have mainly measured Th cell activity in the circulation, because it is technically difficult to quantify CTLs in pigs or to demonstrate T cells in the airways. Proliferating lymphocytes in BALF and blood increased from 7 dpi onwards, and these cells have shown inter-subtypic cross-reaction upon *in vitro* re-stimulation (Heinen *et al.*, 2001a). Pigs infected with the pH1N1 showed activated $CD4^+$ and $CD8^+$ T cells in the peripheral blood on 6 dpi (Lange *et al.*, 2009). Moderate numbers of cells producing IFN-γ have been demonstrated in the porcine nasal mucosa after infection with SIV. Yet, cell numbers were much higher in TBLN and spleen, and they peaked 3 weeks post-infection.

1.3.3 Homologous, heterovariant and heterosubtypic immunity

After primary infection with influenza virus, virus-specific IgA and IgG antibody secreting cells (ASCs) as well as Th and CTLs will be present in the tissues lining the respiratory tract and in the local lymph nodes. The immune system has built up a memory that will lead to a faster, stronger, more localized and more accurate secondary response, upon a second infection with the same or a closely related virus strain. This immunity is called **homologous immunity**. It is primarily mediated by memory B cells and the VN antibodies they produce and can be life-long (reviewed in (Subbarao *et al.*, 2006)). As an example, in 1977, the H1N1 virus first reappeared in the human population since 1957, the people who had been infected 20 years previously with similar H1N1 viruses were still resistant to infection or disease (Fox *et al.*, 1982).

While immunity protects against re-infection with the same or a similar virus, it is less protective or absent when a more distant virus is encountered. This immunity is defined as **heterovariant immunity** when the protection is conferred by previous exposure to a virus of the same HA and NA subtype (i.e. between two H3N2 variants), or **heterosubtypic immunity** when conferred by previously exposure to a virus of another HA and/or NA subtype (i.e. between H3N2 and H1N1/H1N2). Those “broader” protections can occur in the absence of detectable HI antibodies in serum, and antibodies against more conserved viral proteins, especially mucosal antibodies, as well as CMI are likely to contribute to the protection.

Cross-protection between different influenza viruses in rodents, ferrets, and humans

Heterovariant immunity has been demonstrated in mice and ferrets under experimental conditions. Prior infection with a seasonal H1N1 virus from the 1930s-40s protected mice against a lethal challenge with mouse-adapted pH1N1 virus (Skountzou *et al.*, 2010). Likewise, prior infection of ferrets with a seasonal H1N1 virus from the 1930s-40s resulted in a significant reduction in virus titers in nasal turbinates and lungs after challenge with the pH1N1 (O'Donnell *et al.*, 2012). In contrast, prior infection or vaccination with a more recent seasonal H1N1 strain from the 1990s-2000s, which is far less related to pH1N1, provided minimal protection against pH1N1 challenge in mice and ferrets (Laurie *et al.*, 2010; Manicassamy *et al.*, 2010; O'Donnell *et al.*, 2012).

It is believed that partial, but limited cross-protective heterovariant immunity may exist in humans between variants within the same HA and NA subtype, and the extent of cross-protection depends on the antigenic relatedness between viral HA and NA genes. As an illustration, elderly people who have been exposed to historic human seasonal H1N1 viruses prior to 1950s had pre-existing antibodies against the pH1N1 virus and were relatively spared from contracting infections and developing disease in the 2009 H1N1 pandemic (Hancock *et al.*, 2009; Ikonen *et al.*, 2010; Reed & Katz, 2010; Yu *et al.*, 2008). In contrast, the pH1N1 attack rates were highest in young adults and children, even though they had substantial pre-existing antibodies and CMI to seasonal H1N1 viruses from the 2000s (Skowronski *et al.*, 2011). Human seasonal H1N1 and H3N2 viruses continuously undergo antigenic drift, and immunity to previous strains may not be able to provide sufficient protection to the new emerged variant strains. For example, an A/Fujian/411/2002-like virus caused an unusually severe 2003–2004 flu season in humans. Only two amino acid substitutions in HA (H155T, Q156H) were sufficient for this A/Fujian/411/2002-like virus to become antigenically distinct from the precursor A/Panama/2007/1999-like virus (Jin *et al.*, 2005). As a result of the rapid antigenic drift of seasonal strains, the seed strains in influenza vaccines are updated every 2-3 years to ensure their protective efficiency in humans (Hay *et al.*, 2001; Russell *et al.*, 2008).

Heterosubtypic immunity has also been repeatedly reported in laboratory animals. While such immunity usually does not provide sterilizing immunity (i.e. prevent infection), it may clearly reduce virus replication and mortality. Schulman and

Kilbourne showed that prior infection with an H1N1 strain protected mice from subsequent challenge with an otherwise lethal dose of H2N2 virus (Schulman & Kilbourne, 1965). Many subsequent studies have confirmed and extended those observations. For example, prior infection of mice or cotton rats with H1N1 or pH1N1 did not prevent the subsequent H3N2 challenge infection, but virus clearance was accelerated in the nose and lung (Hillaire *et al.*, 2011; Kreijtz *et al.*, 2007; Straight *et al.*, 2006). Reductions in both shedding and transmission of pH1N1 have been demonstrated in guinea pigs previously exposed to seasonal H3N2 viruses (Steel *et al.*, 2010). In ferrets, prior infection with pH1N1 or a seasonal H1N1 virus resulted in a 1-2 days shorter duration of nasal excretion after challenge with a seasonal H3N2 virus (Laurie *et al.*, 2010; Yetter *et al.*, 1980).

Heterosubtypic immunity is much debated in humans. A report on cross-subtype protection in humans during either concurrent or successive H3N2 and H1N1 epidemics in Japanese high schools revealed that a significantly smaller proportion of students were infected with H1N1 following an H3N2 epidemic compared to students without previous exposure to the H3N2 virus (Sonoguchi *et al.*, 1985). However, previous infection of children <3 years old with H1N1 or H3N2 influenza provides little protection against experimental challenge with attenuated heterosubtypic strains (Steinhoff *et al.*, 1993). Similarly, at the start of the 1957 pandemic, when H2N2 viruses spread through a population in which H1N1 influenza was endemic, there was no evidence of cross-protection of children, although there was evidence of partial heterosubtypic protection of adults with extensive past influenza exposure (Epstein, 2006).

Compared to the long-lasting homologous immunity, heterosubtypic immunity is usually short-lived. Liang *et al* used the mouse model to demonstrate that heterosubtypic immunity in the nose began to disappear at about 4-5 months post-infection, but it remained in the lung for over 7 months (Liang *et al.*, 1994). In humans, memory T cells to influenza, particularly $CD8^+$ CTL activities, also wane over time, with a half-life of 2-3 years (McMichael *et al.*, 1983). This may explain the generally weaker heterosubtypic immunity observed in humans than in laboratory animals, as experimental studies are performed with several weeks between infections, while most human influenza infections have an interval of several years. Because memory B- and T- cells decline over time, experimental studies tend to overestimate the extent of cross-protection between viruses in the field.

Cross-protection between different influenza viruses in pigs

In pigs, infection with SIV of one subtype may confer at least partial heterovariant protection from subsequent infections (reviewed in (Van Reeth & Ma, 2013)). For example, a nearly complete protection has been observed between North American α- and γ-cluster H1 SIVs (Vincent *et al.*, 2008a), and between a European H1avN1 SIV and a North American triple-reassortant H1N1 SIV (De Vleeschauwer *et al.*, 2011) as well as the pH1N1 virus (Busquets *et al.*, 2010), despite up to 20-30% amino acid sequence differences in the HA1 of the priming and challenge viruses. In these studies, the challenge virus was either undetectable in nasal swabs or lung tissues of these previously infection-immune pigs, or virus isolation rates and viral titers were significantly reduced compared to those in challenge controls. Cross-protection between SIVs of different subtypes (heterosubtypic protection) was much weaker. Prior infection with European H1avN1 could not prevent the subsequent infection of a European H1huN2 (Van Reeth *et al.*, 2003), or a European H3N2 SIV (Heinen *et al.*, 2001a), but nasal shedding was on average 1-2 days shorter than in challenge control pigs. In all of the above studies, cross-protection occurred in the absence of cross-reactive HI antibodies in the serum, but there are some indications for the protective role of cross-reactive serum VN and/or NI antibodies. Antibodies in nasal washes and BALF, and CMI responses may also contribute to the broad protection, but their relative contributions as well as the target viral proteins remain to be identified.

1.4 The pig as a model for influenza compared to mice and ferrets

Influenza in pigs has gained significant interest since the 2009 swine-origin H1N1 pandemic, as well as the use of pigs as a model for human influenza. Pigs are physiologically, anatomically, and immunologically similar to humans (Lefevre *et al.*, 2012). In contrast to the mouse lung, the porcine lung has marked similarities to its human counterpart in terms of the structure of the tracheobronchial tree, lung physiology, airway morphology, abundance of airway submucosal glands, and patterns of glycoprotein synthesis (Goco *et al.*, 1963; Ibrahim *et al.*, 2006). Pigs can be naturally infected by a wide range of both avian and human viruses, and the clinical manifestations and pathogenesis after infection with swine-adapted influenza viruses closely resemble those of human influenza (Van Reeth *et al.*, 2012). This

close similarity has recently also been confirmed for pH1N1 virus (Brookes *et al.*, 2010; Lange *et al.*, 2009). In humans and swine, virus replication occurs mainly locally in the upper and lower respiratory tract, and the cell receptor distributions with respect to α2,6-and α2,3-linked galactose are strikingly similar in them (van Riel *et al.*, 2007). In both species, α2,6-linkages predominate in the upper respiratory tract, whereas α2,3-linked galactose is restricted to the epithelium of the lower respiratory tract. This results in efficient replication of viruses with human-like but not avian-like receptor specificity in the epithelial cells of the upper respiratory tract (Van Poucke *et al.*, 2010). This may at least partially explain the relatively high resistance of both pigs and humans to infection with AIVs such as the H5N1 subtype (Lipatov *et al.*, 2008). Ferrets, in contrast, are highly susceptible to H5N1 infections (Zitzow *et al.*, 2002). In ferrets, seasonal viruses predominantly replicate in the upper respiratory tract, and alveolar infection and pneumonitis are less pronounced (Maher & DeStefano, 2004; Smith & Sweet, 1988). Mice, on the other hand, are not natural hosts for influenza viruses and infection typically leads to lethal pulmonary infection (Bouvier & Lowen, 2010). Also, many clinical signs of influenza virus infection in humans are not present in mice (Bouvier & Lowen, 2010; Thangavel & Bouvier, 2014). Furthermore, viruses generally require prior adaptation in order to achieve productive infection and clinically apparent signs of disease. The adapted virus may eventually become antigenically and/or phenotypically very different from the initial strain (Margine & Krammer, 2014).

Although mice and ferrets have been most frequently used to study protective immunity against influenza, especially for human influenza vaccine evaluation, the limitations of those models are obvious (Table 2). The immune responses vary in different mouse strains (Srivastava *et al.*, 2009), and protecting mice against influenza virus is much easier than natural influenza virus hosts like pigs (Dormitzer *et al.*, 2011). The course of influenza virus infection and pathogenesis in ferrets closely resembles that in humans (Belser *et al.*, 2011; Thangavel & Bouvier, 2014; van der Laan *et al.*, 2008), but influenza seronegative ferrets are extremely difficult to obtain. Another limitation of the ferret model is the lack of specific reagents for studying the ferret immune system. The immune responses in pigs are remarkably similar to those in humans, and the cytokine profiles in BALF of SIV-infected pigs are also identical to those observed in nasal lavage fluids of experimentally infected humans (Hayden *et al.*, 1998; Van Reeth *et al.*, 1998). The advantage of pigs over

the ferret model is the availability of more immunological reagents and methods (Summerfield, 2009), including extensive information on swine leukocyte antigens.

Table 2. Summary of the advantages and disadvantages of different laboratory animal models to study immunity against influenza viruses (Margine & Krammer, 2014)

Species	Advantages	Limitations
Mice	• Small size, easy to hold • Low cost (animals, housing) • Homogeneous responses-inbred, pathogen free • Availability of molecular biology/immunology reagents • Pathology of viral pneumonia caused by highly pathogenic viruses (1918 H1N1, HPAI H5N1) similar to humans	• Seasonal influenza virus strains need prior adaptation in order to achieve efficient replication and virulence • Respiratory tract anatomy and receptor distribution different from humans • None human-like clinical signs; use surviving rates and weight loss as the readout of infection/protection • Not suitable for transmission experiments • Susceptibility to influenza viruses and immune responses vary in different mouse strains • Not able to evaluate nasal virus shedding
Ferret	• Respiratory tract anatomy and receptor distribution similar to humans • Human and avian influenza virus isolates replicate without prior adaptation • Human-like clinical signs and pathology of disease • Suitable for transmission experiments	• Limited availability of molecular biology/immunology reagents • Difficult to obtain influenza naïve ferrets • Response variability among individuals • Systemic disease different from in humans • Very expensive
pig	• Respiratory tract anatomy and receptor distribution similar to humans • Natural host of various H1 and H3 influenza viruses • Some human and avian influenza virus isolates replicate without prior adaptation • Human-like clinical signs and pathology of disease • Availability of large amounts of tissues for various analysis • Availability of molecular biology/immunology reagents	• Response variability among individuals • Difficult to obtain influenza naïve pigs • Practical issues-big size, husbandry requirements

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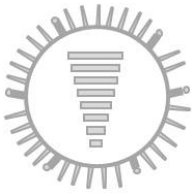


Chapter 2

Aims of the thesis

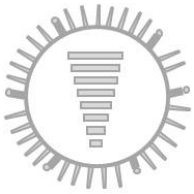
1. Most, if not all swine influenza viruses (SIVs) of H3N2 subtype have derived their hemagglutinin (HA) and neuraminidase (NA) proteins from H3N2 viruses that once circulated in the human population. But after their transmission to and adaptation to swine, these viruses have followed distinct evolutionary pathways than their human precursors. The evolution of European H3N2 SIVs from 1984 to 2002 has been described by de Jong et al (de Jong *et al.*, 1999; de Jong *et al.*, 2007), but there is no information about the more recent viruses. We therefore aimed to characterize the genetic and antigenic evolution of European H3N2 SIVs isolated between 1998 and 2012, and to compare it with that of North American swine and human H3N2 viruses in the same time period.

2. The genetic and antigenic properties of SIVs are distinct in different continents, and the introduction of a novel “exotic” SIV may pose a threat to swine populations. The H3N2 variant or H3N2v is a North American swine-origin virus, in which all but the M gene segments differ from those of European H3N2 SIVs. The virus is also of public health concern, because it has caused 343 zoonotic infections between 2011 and 2014. We have examined the extent of cross-protection against this virus in pigs previously infected with the European H3N2 SIV and the associated immune mechanisms.

3. The 2009 pandemic H1N1 virus (pH1N1) has become established in swine shortly after the start of the 2009 pandemic and it is co-circulating with previously established SIVs in swine populations worldwide. We aimed to study the extent of cross-protection between different European SIV lineages, more specifically 1) between pH1N1 and three different European H1 SIV lineages, and 2) between these H1 viruses and a European H3N2 SIV. Because the pH1N1 is a shared virus between humans and swine, and pigs are a valuable animal model for human influenza, this study may give indications as to whether the high population immunity to pH1N1 in humans may offer some protection against infections with previously established SIVs.

4. The pH1N1 virus contains the HA from the historical 1918 pandemic H1N1 virus, which has disappeared from the human population for decades. This virus has been maintained in the swine reservoir and it has ultimately reassorted with other

SIVs to give rise to the pH1N1 virus. H3N2 SIVs also contain historical human-like HAs and NAs, so that similar events could occur with these viruses in the future. Therefore, H3N2 viruses of swine have recently been added to the WHO pandemic preparedness list. The pandemic risk of H3N2 SIVs is determined in part by the extent to which pre-existing immunity to human seasonal H3N2 viruses can cross-protect against H3N2 viruses of swine. We first used pigs as a model for humans to assess whether immunity resulting from infection with an old (1975) or a more recent (2005) human seasonal H3N2 virus protects against a European H3N2 SIV after experimental challenge. Subsequently, we have examined sera from people of various age categories for the prevalence of antibodies against European and North American swine-origin H3N2 viruses, as well as human seasonal H3N2 viruses of various decades.



Chapter 3

Genetic and antigenic evolution of European H3N2 swine influenza viruses during 1998-2012

Yu Qiu & Kristien Van Reeth

Manuscript in preparation

3.1 Abstract

Swine influenza viruses (SIVs) of H3N2 subtype have become established in European pigs since 1984, and they are double reassortants derived from human seasonal H3N2 viruses and European H1N1 SIVs. Analyses of European H3N2 SIVs during 1998-2012 revealed that their hemagglutinin (HA) and neuraminidase (NA) nucleotide sequences changed over time, but the amino acid sequences in antigenic regions remained largely conserved, especially the immunodominant sites. Consistent with the genetic evolution, antigenic analyzes using the hemagglutination-inhibition (HI) assay and neuraminidase-inhibition (NI) assay show European H3N2 SIVs have remained in antigenic stasis since 1998. We compared our data with the genetic and antigenic evolution of H3N2 viruses in North American swine and humans.

3.2 Introduction

Since the Hong Kong pandemic in 1968, the H3N2 pandemic virus and its descendants have not only been circulating in humans, but they were also isolated from pigs (Shortridge *et al.*, 1979; Shortridge *et al.*, 1977). In Europe, H3N2 viruses were occasionally isolated in pigs in 1970s (Tumova *et al.*, 1980), but they have only become established in the mid 1980s after reassorting with endemic H1N1 swine influenza viruses (SIVs). Those established H3N2 SIVs contain human-like hemagglutinin (HA) and neuraminidase (NA) genes, whereas all internal genes are of avian-like H1N1 SIV origin (Campitelli *et al.*, 1997; Castrucci *et al.*, 1993). Genetic analysis of the HA1 segments reveals that those H3N2 SIVs are most related to human seasonal H3N2 viruses in 1973-75 (de Jong *et al.*, 2007; Qiu *et al.*, 2015; Qiu *et al.*, 2013). Previous studies by de Jong *et al* report the occurrence of considerable antigenic drift in H3N2 SIVs (de Jong *et al.*, 2007; de Jong *et al.*, 1999). The authors also classified European H3N2 SIVs into cluster 1 viruses (1984-1993) and cluster 2 viruses (1993-2002), with the latter showing reduced cross-reactivity with A/Port Chalmers/1/73 in the hemagglutination-inhibition (HI) assay. Based on these, the authors recommended a replacement of the A/Port Chalmers/1/73 strain in the SIV vaccine. Our previous study has shown a A/Port Chalmers/1/73-based commercial vaccine induced similar high HI antibody titers against European H3N2 SIVs from 1998-2012 in pigs, which were 2- or 4- fold lower than the homologous A/Port Chalmers/1/73 titer (De Vleeschauwer *et al.*, 2015). Those vaccinated pigs showed

significantly reduced lung virus titers after experimental challenge with a European H3N2 SIV sw/Gent/172/08. A similar strong protection induced by A/Port Chalmers/1/73-based commercial vaccine has also been observed after challenge of pigs with the earlier European H3N2 SIV strain sw/Fanders/1/98 or sw/Oedenrode/7C/96 (Heinen *et al.*, 2001; Van Reeth *et al.*, 2001).

In North America, pigs were free of H3N2 viruses until 1998, when triple-reassortant H3N2 viruses containing gene segments from the classical swine virus (NP, M, NS), H3N2 human seasonal influenza virus (PB1, HA, NA) and avian influenza virus (PB2, PA) became successfully established in the pig population (Webby *et al.*, 2000; Zhou *et al.*, 1999). This genome composition of SIV is referred to as the triple-reassortant internal gene (TRIG) cassette (Vincent *et al.*, 2008). Those TRIG H3N2 SIVs have been grouped into I-III clusters, as a result of three separate human seasonal H3 gene introductions in 1995 (H3-I), 1997 (H3-II), and 1996 (H3-III), respectively (Webby *et al.*, 2000). Low cross-reactivity was reported among clusters (Leuwerke *et al.*, 2008; Richt *et al.*, 2003). Swine H3 cluster IV evolved from cluster III and was first reported in 2005 (Olsen *et al.*, 2006), but remained relatively stable until 2010 (Kitikoon *et al.*, 2013a). The introduction of the 2009 pandemic H1N1 virus (pH1N1) and reassortment with previously established SIVs including H3N2 altered both the genotype and phenotype of H3-IV viruses. At least 10 H3N2 genotype patterns have been identified in the United State (US) since August 2011 (Kitikoon *et al.*, 2013b).

In spite of H3N2 SIVs circulating in both Europe and North America have their HA derived from human seasonal H3N2 viruses, the HAs seem to drift more slowly in pigs than in humans. In North America, TRIG H3N2 SIVs have shown antigenic drift in HA during 1998-2013, albeit less intensive compared to their human counterparts (Lewis *et al.*, 2014). The HA antigenic evolution of European H3N2 SIVs during 1982-2002 occurred at a rate approximately six times slower than the rate in human viruses (de Jong *et al.*, 2007). But knowledge about the more recent virus isolates after 2002 remains limited until the present study. Also, NA is another important antigenic determinant of influenza viruses besides HA, but antigenic characterization of NA is limited, especially in SIV research. In the present study, we aimed to investigate the genetic and antigenic evolution of both HA and NA from European H3N2 SIVs, and mainly focus on isolates in 1998-2012. We also aimed to compare such evolution with those of human seasonal and North American swine H3N2 viruses, where possible.

3.3 Materials and methods

Sequences of HA1 and NA genes of sw/Gent/1/84, and all available sequences in Genbank from European H3N2 SIVs during 1998-2012 were used for studying genetic evolution. To calculate the average rate of nucleotide substitutions in HA1 or NA, we measured the genetic distance in the Maximum-likelihood (ML) phylogenetic tree from sw/Gent/1/84 to each isolate. The sw/Gent/1/84 virus is one of the first human-like reassortant H3N2 SIVs isolated in Europe, and is assumed to be the ancestor virus for European H3N2 SIVs. In addition, we compared the HA1 and NA amino acid sequences of sw/Gent/1/84, commercial vaccine seed strains A/Port Chalmers/73, sw/Belgium/220/92, and sw/Bakum/1769/03, and a selection of H3N2 SIVs circulating from 1998 to 2012. Residue differences at putative antigenic sites of the HA1 and NA, as defined by others (Colman *et al.*, 1983; Underwood, 1982; Wiley *et al.*, 1981), were identified by alignment using MEGA 5.05 software (http://www.megasoftware.net/).

To study the antigenic evolution of H3N2 SIVs, HI and neuraminidase-inhibition (NI) assays using hyperimmune or post-vaccination swine sera were performed as previously described (Sandbulte *et al.*, 2009; Van Reeth *et al.*, 2003). Hyperimmune swine sera were raised against each of 8 representative European H3N2 SIV isolates from different years. The pigs were inoculated intranasally with live influenza viruses, three weeks later they were injected intramuscularly (2 ml) and intradermally (1 ml) with the same virus in combination with complete Freund's adjuvant. Hyperimmune sera were collected when the homologous HI titer was ≥320. Post-vaccination sera were prepared in previous studies with commercial inactivated whole virus vaccines (De Vleeschauwer *et al.*, 2015; Kyriakis *et al.*, 2010). Sera were collected at two weeks after the booster vaccination. Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete inhibition of HA of 4 hemagglutinating units of virus (HI assay), or 50% reduction of NA activity (NI assay). Starting dilutions were 1:10 in both assays.

3.4 Results and discussions

3.4.1 Genetic evolution

The genetic evolution in terms of nucleotide substitutions of H3N2 SIVs seemed to have occurred at similar rates in HA1 and NA (Figure 1). At the nucleotide level,

H3N2 SIVs have evolved on average at a rate of change in ML distance of 0.0035 and 0.0023 per year (~3.5 and 3.4 nucleotide substitutions per year) in HA1 and NA, respectively, from 1998 to 2012. Similar substitution rates of HA1 were observed in human and European swine H3N2 viruses during 1982-2002 (de Jong *et al.*, 2007), as well as in human and North American swine H3N2 viruses during 1998-2012 (Lewis *et al.*, 2014).

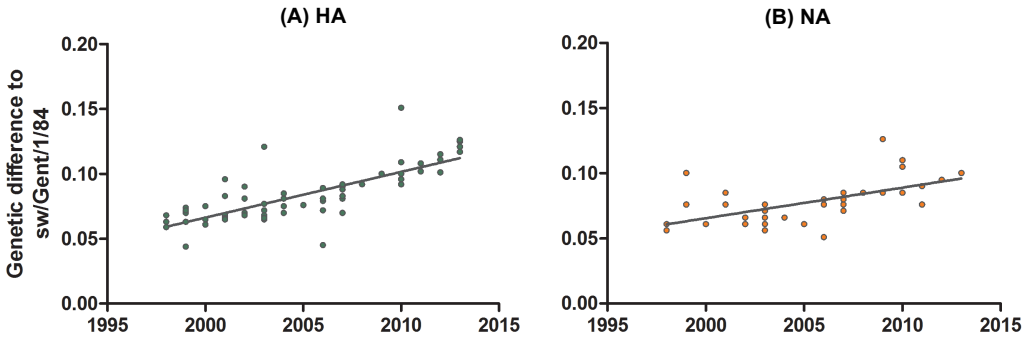


Figure 1. Rates of genetic evolution of hemagglutinin (A) and neuraminidase (B) of European H3N2 swine influenza viruses during 1998-2012. The genetic distance to the ancestor virus sw/Gent/1/84 was calculated from the maximum-likelihood phylogenetic tree, plotted as a function of time. The slope gives the rate of evolution of nucleotide substitutions per year. The regression lines for panels A and B had slopes of 0.0035 and 0.0023, respectively.

Amino acid differences at presumed antigenic sites of the HA are shown in Figure 2. SIVs during 1998-2012 evolved to differ from the ancestral virus sw/Gent/1/84 by six amino acids in all five antigenic sites: 53, 137, 145, 217, 220 and 278. The sequences in immunodominant antigenic site A and B were almost identical in those SIVs. In contrast, during the same time period North American swine and human H3N2 viruses have obtained as many as 7 and 8 residue substitutions in sites A and B, respectively (Lewis *et al.*, 2014). Those viruses also had close relatedness in the antigenic sites of the NA (data now shown).

3.4.2 Antigenic evolution

The antigenic evolution of H3N2 SIVs during 1998-2012 was characterized by cross-HI and cross-NI assays (Table 1 and 2). Those viruses have shown similar strong cross-reactions to hyperimmune swine sera against sw/Gent/1/84 as well as to all three post-vaccination swine sera. This indicates little antigenic drift occurred in HA

or NA during virus evolution, and is in good agreement with the observed few changes in antigenic sites and with previous challenge studies (De Vleeschauwer *et al.*, 2015; Heinen *et al.*, 2001; Van Reeth *et al.*, 2001), but it disagrees with the previous conclusion that H3N2 SIVs have experienced significant antigenic drift during 1982-2002 (de Jong *et al.*, 2007; de Jong *et al.*, 1999). However, their conclusion was challenged by the fact that in the same study, ferret anti-sera against A/Victoria/3/75, sw/Brisbane/1/84 or sw/Ommel/97 discriminated the two clusters less well as compared to anti-A/Port Chalmers/1/73 sera.

During 1998-2012, North American H3N2 SIVs seemed to have experienced more antigenic drift in the HA1 than their European counterparts. As an illustration, Kitikoon *et al* have shown that hyperimmune swine sera against contemporary swine H3 isolates after 2010 had low cross-reactivity to the old cluster I virus (sw/Texas/4199-2/1998), older cluster IV isolates (sw/Minnesota/01146/2006) and inconsistent cross-reactivity among the recent isolates (Kitikoon *et al.*, 2013b; Lewis *et al.*, 2014). A similar comparison for the NA was not possible because the NA antigenic evolution of North American H3N2 SIVs has not been studied yet. Human seasonal

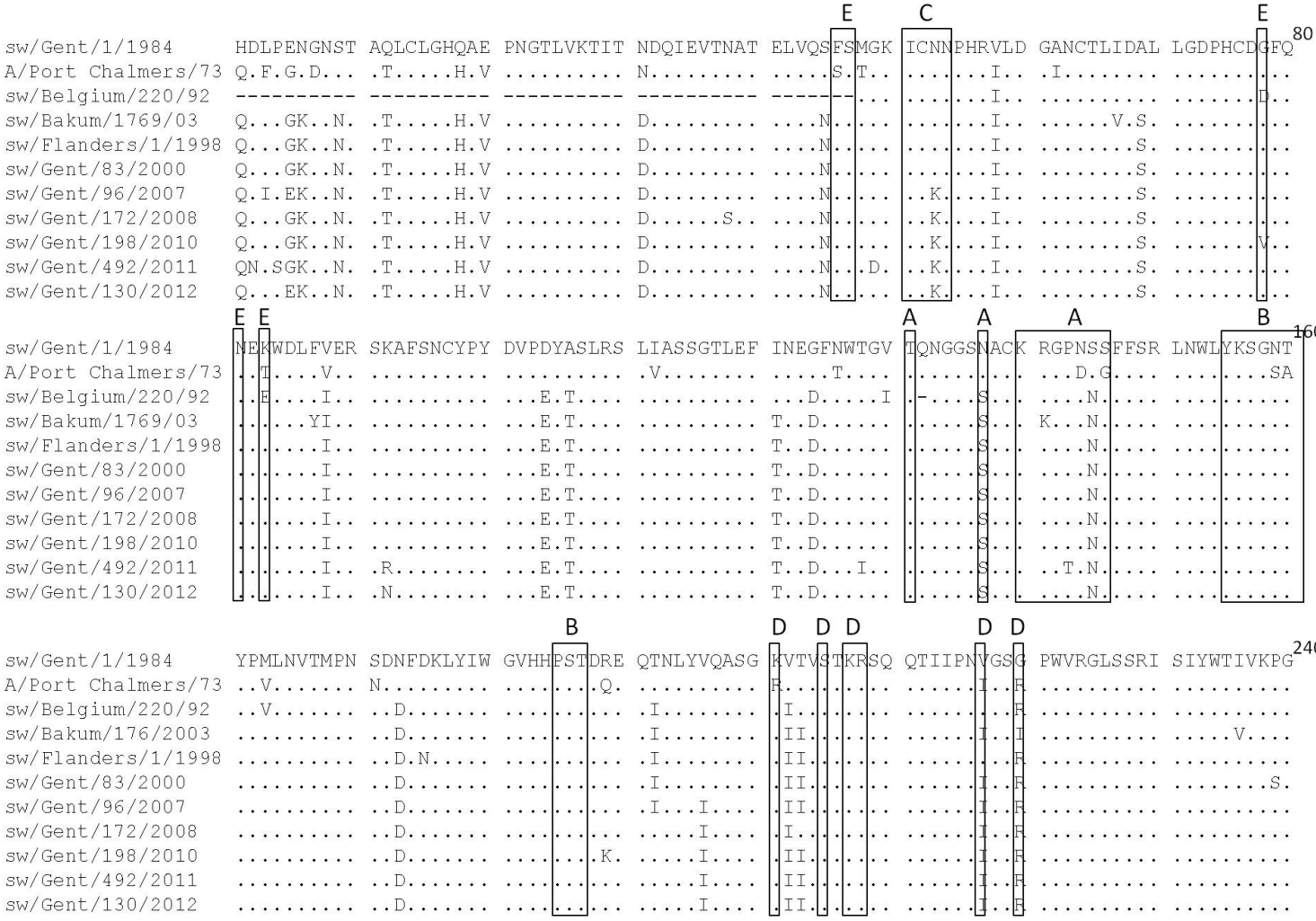


Figure 2. Alignment of deduced amino acid sequences in the HA1 of European H3N2 swine influenza viruses. Residues in the open boxes represent previously identified antigenic sites (A, B, C, D, and E) of H3. Only amino acids different from those in the sw/Gent/1/84 sequence are shown, conserved residues are shown as dots. Unknown amino acids are represented as dashes.

Table 1. Hemagglutination-inhibition (HI) reactions of post-vaccination or hyperimmune swine sera with various H3N2 viruses

	Post-vaccination swine serum			Hyperimmune swine serum						
Virus	A/Port Chalmers/ 1/73	sw/Belgium/ 220/92	sw/Bakum/ 1769/03	sw/Gent/ 1/84	sw/Flanders/ 1/98	sw/Gent/ 83/00	sw/Gent/ 80/01	sw/Gent/ 131/05	sw/Gent/ 172/08	sw/Gent/ 130/12
Vaccine strain										
A/Port Chalmers/73	640	20	10	320	640	80	20	320	160	40
sw/Belgium/220/92	320	160	40	640	2560	320	160	1280	1280	160
sw/Bakum/1769/03	320	160	80	1280	5120	640	160	2560	2560	80
Field strain										
sw/Gent/1/84	1280	160	40	5120	5120	640	160	5120	2560	320
sw/Flanders/1/98	320	160	40	640	1280	640	160	1280	1280	160
sw/Gent/83/00	320	160	40	640	5120	640	160	2560	2560	320
sw/Gent/80/01	320	320	80	1280	5120	640	320	2560	2560	320
sw/Gent/131/05	640	320	80	1280	5120	640	320	2560	2560	640
sw/Gent/96/07	640	320	80	1280	5120	640	160	2560	2560	320
sw/Gent/172/08	640	320	80	1280	5120	320	160	2560	2560	320
sw/Gent/538/10	320	160	40	1280	5120	320	160	2560	2560	320
sw/Gent/205/11	160	80	40	640	10240	160	80	5120	5120	320
sw/Gent/130/12	640	320	80	1280	5120	640	320	5120	5120	640

Titers against the homologous virus are underlined

Table 2. Neuraminidase-inhibition (NI) reactions of post-vaccination or hyperimmune swine sera with various H3N2 viruses

	Post-vaccination swine serum			Hyperimmune swine serum						
Virus	A/Port Chalmers/ 1/73	sw/Belgium/ 220/92	sw/Bakum/ 1769/03	sw/Gent/ 1/84	sw/Flander/ 1/98	sw/Gent/ 83/00	sw/Gent/ 80/01	sw/Gent/ 131/05	sw/Gent/ 172/08	sw/Gent/ 130/12
Vaccine strain										
A/Port Chalmers/73	2560	40	10	2560	1280	320	80	320	320	80
sw/Belgium/220/92	320	640	160	2560	5120	320	80	2560	5120	1280
sw/Bakum/1769/03	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Field strain										
sw/Gent/1/84	640	160	40	2560	2560	320	80	1280	640	320
sw/Flanders/1/98	640	640	160	2560	5120	1280	640	2560	2560	1280
sw/Gent/83/00	640	640	160	1280	5120	640	80	2560	5120	1280
sw/Gent/80/01	640	640	80	1280	2560	640	320	2560	5120	2560
sw/Gent/131/05	640	640	160	1280	5120	640	320	2560	5120	1280
sw/Gent/96/07	320	320	40	2560	5120	1280	640	2560	5120	1280
sw/Gent/172/08	320	640	80	2560	5120	1280	640	2560	5120	1280
sw/Gent/538/10	640	640	160	2560	5120	160	80	2560	5120	2560
sw/Gent/205/11	640	640	80	1280	5120	640	320	2560	5120	2560
sw/Gent/130/12	320	640	160	1280	5120	640	160	2560	5120	5120

Titers against the homologous virus are underlined. n.a.: not available

H3N2 viruses seemed to have the most prominent antigenic drift in HA1 compared to their European and North American swine counterparts, as human seasonal strains tend to have a significantly reduced or undetectable HI titer to a strain isolated 3-5 years apart (Hay *et al.*, 2001; Russell *et al.*, 2008). A significant antigenic drift has also been observed in NAs of human seasonal H3N2 viruses. For example, Sandbulte *et al* used ferret post-infection antisera to study the NA antigenic evolution of human seasonal H3N2 viruses during 1968-2007 (Sandbulte *et al.*, 2011). In that study, cross-NI reactions within human seasonal H3N2 viruses were asymmetrical: some antisera against old strains have lost cross-reactivity with more recent strains, whereas sera against more recent viruses often retained variable cross-reactivity with the old strains.

The differences in the antigenic drift rates in the three H3N2 virus lineages may be due to a different host immune pressure. Seasonal influenza occurs globally with an annual attack rate estimated at 5%-10% in adults and 20%-30% in children (WHO). Human H3N2 viruses require frequent antigenic changes in the HA to ensure that a sufficiently large pool of immunologically susceptible hosts is available. In pigs, because of the short average life span (~ 6 months) and the continuous renewal of the pig population, influenza virus evolution may experience less immune pressure in pigs than in humans. But the vaccination coverage differences in European and North American population may have an impact on the different viral drift rates. It is reported in the US, 70% of large producers vaccinated breeding females and approximately 20% vaccinated weaned pigs in 2006 (Vincent *et al.*, 2008). In Belgium, however, SIV vaccines are used in <20% of breeding females and rarely used for weaned pigs. Moreover, in contrast to Europe, live transport of millions of pigs is very common in swine farming in the US, which may promote the dissemination and evolution of SIVs (Nelson *et al.*, 2015). Furthermore, the several separate introductions of the human-like H3 HAs into North American swine, and the frequent reassortment events between H3N2 SIVs and other influenza viruses may contribute to the more antigenic diversity of H3N2 SIVs in North America compared to that in Europe.

3.4.3 Conclusions

Taken together, we found that European H3N2 SIVs from 1998-2012 remained in an antigenic stasis and retained high relatedness to the prototype virus sw/Gent/1/84.

More significant antigenic drift has been observed in North American swine and human H3N2 viruses, even though the nucleotide substitution rates were similar in all three lineages. Based on our observation, we assume that there is not an urgent need for updating the H3N2 component in European swine influenza vaccine. However, like in the US, the epidemiology of European SIVs has become more complex after the establishment of pH1N1 in pigs, and reassortants between those two lineages have been isolated and some are now common in certain regions (Chiapponi *et al.*, 2014; Simon *et al.*, 2014). Genetic and antigenic characterization of those newly emerged reassortant H3N2 SIVs and surveillance should be continued to warrant a broad knowledge of H3N2 SIVs in Europe.

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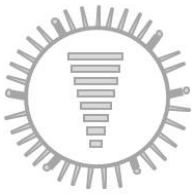

Chapter 4

Study of cross-protection between swine influenza viruses in pigs

Chapter 4.1

Infection of pigs with a European H3N2 swine influenza virus induces cross-protective immune mechanisms against a North American H3N2 variant virus

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Manuscript in preparation

4.1.1 Abstract

The swine-origin H3N2 variant (H3N2v) influenza viruses, which have infected 343 humans in the United States (US) since August 2011, are genetically and antigenically distinct from the H3N2 swine influenza viruses (SIVs) circulating in Europe. Although the H3N2v virus has so far never been isolated in Europe, it may be introduced by export of pigs from the US to other continents. This study was designed to examine to what extent pigs previously infection with a European H3N2 SIVs would be protected against a H3N2v challenge infection. Influenza naïve pigs were first inoculated intranasally with a European H3N2 SIV and 8 weeks later followed with a H3N2v virus. Infection with the European H3N2 SIV induced minimal H3N2v virus-neutralization (VN) antibodies, and no hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) antibodies in serum. Cross-reactive IgA and IgG antibody secreting cells (ASCs) and IFN-γ secreting cells (SCs) specific to H3N2v were demonstrated, especially in nasal mucosa. Upon H3N2v challenge, the magnitude and duration of virus excretion and virus titers in respiratory tissues at day 3 post-challenge were significantly reduced in European H3N2 SIV-inoculated pigs compared to previously uninoculated challenge control pigs. This protection was associated with vigorous IgG and IgA ASC boost responses to H3N2v, which were by far most pronounced in the nasal mucosa. Our data show partial protection between H3N2 viruses of two distinct lineages, and support the notion that the pig is a valuable model to study mucosal immune responses and to improve our understanding of broad-spectrum immunity to influenza.

4.1.2 Introduction

Both European and North American swine influenza viruses (SIVs) have their hemagglutin (HA) and neuraminidase (NA) derived from historical human seasonal H3N2 viruses, but their origin and genetic constellations are different. In Europe, H3N2 SIVs are derived from descendants of the A/Hong Kong/1/68 pandemic H3N2 virus which crossed the species barrier to pigs in the 1970s, but they have evolved further through genetic reassortment with the endemic avian-like H1N1 SIV. This has resulted in H3N2 SIVs with human-like HA and NA genes and avian-like internal genes (Campitelli *et al.*, 1997; Castrucci *et al.*, 1993). In North America, H3N2 viruses only appeared in swine since 1998. They are known as triple-reassortant viruses because their HA, NA and polymerase B1 genes are derived from human

seasonal H3N2 viruses from the 1990s, and the remaining internal genes are of avian influenza virus and classical H1N1 SIV origin (Webby *et al.*, 2000). Since 2009, novel reassortant H3N2 viruses with variable numbers of internal genes derived from the 2009 pandemic H1N1 (pH1N1) virus have been reported frequently and this has further complicated the epidemiology of swine influenza in the US (Kitikoon *et al.*, 2013). In 2009-2012, these novel pH1N1 reassortants accounted for 54% of H3N2 SIVs isolated (Kitikoon *et al.*, 2013). Reassortant viruses with seven genes from the "triple-reassortant" H3N2 SIVs and only the matrix (M) gene from the pH1N1 virus have become one of the dominant genotypes (Kitikoon *et al.*, 2013). These viruses, called "H3N2 variant" or "H3N2v" when isolated from humans, have caused 343 human infections in the US from July 2011 through October 2014 (http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm). This H3N2v virus and related North American H3N2 SIVs have not yet been isolated from Europe. This raises the question as to whether pigs immune to European H3N2 SIVs would be protected against infection with the antigenically and genetically distinct H3N2v if the latter virus was introduced in Europe.

Infection with a live H1N1 or H3N2 influenza virus in pigs may provide potent and broad cross-protective immunity against another strain of the same HA and NA subtype (heterovariant protection), despite of the absence of cross-reactive hemagglutination-inhibition (HI) antibodies in serum. For example, pigs that had been previously infected with a historical human seasonal H3N2 virus in 1975 were completely protected against challenge infection with a European H3N2 SIV (Qiu *et al.*, 2013). In similar experiments, a prior infection of European H1N1 SIV protected pigs against challenge with the pH1N1 (Busquets *et al.*, 2010) or with a North American H1N1 SIV (De Vleeschauwer *et al.*, 2011). Knowledge about what constitutes protective immunity in pigs is sparse, although there is some indication that the nasal wash IgA antibodies target to the challenge virus are more related to broaden immunity than serum IgG antibodies (Loving *et al.*, 2013). In mice and humans, where immune mechanisms are much more extensively studied, IgA and IgG antibodies in the respiratory tract and interferon-γ (IFN-γ) responses contribute to the heterovariant protection against influenza (Clements *et al.*, 1986; He *et al.*, 2015; Liew *et al.*, 1984; Renegar *et al.*, 2004; Tamura *et al.*, 2005; Wagner *et al.*, 1987). Interestingly, the relative importance of each immune component in this process is controversial, and they seem to be partially redundant, since high degrees of

protection were also observed in B cell or IFN-γ deficient mice (Bot *et al.*, 1998; Mozdzanowska *et al.*, 2000).

In pigs, HA-specific antibody responses in respiratory secretions, bronchoalveolar lavage fluid (BALF), and sera have been detected post SIV infection (Lee *et al.*, 1995). Larsen *et al* have used enzyme-linked immunosorbent spot (ELISpot) assays to demonstrate infection with SIV could induce virus-specific IgA and IgG antibody secreting cells (ASCs) in nasal mucosa that peaked on day 21 post inoculation with a North American H1N1 SIV (Larsen *et al.*, 2000). In the same study, virus-specific IFN-γ secreting cells (ISCs) have been detected in spleen, tracheobronchial lymph nodes (TBLNs) and nasal mucosa by the ELISpot assay, which reached a peak on day 21 post-inoculation. However, how those antibodies, ASCs and ISCs are associated with cross-protection remains undefined. In this study, we aimed to investigate to what extent prior infection with a European H3N2 SIV confers protection against H3N2v challenge infection in pigs, as well as the nature of protective immunity. Cellular and humoral immune responses were examined systemically and at respiratory tract mucosal surfaces: antibody titers were measured in serum, nasal secretions and BALF by virus-neutralization (VN), or together with HI and neuraminidase-inhibition (NI) assays, and virus-specific IgG and IgA ASC as well as ISC responses in airway and peripheral blood were determined by ELISpot assays.

4.1.3 Materials and methods

4.1.3.1 Viruses

Viruses used for pig inoculation were sw/Gent/172/08 (sw/Gent/08) and A/Indiana/08/11 (A/IN/11), which represent European H3N2 SIVs and North American H3N2v viruses, respectively. Those two viruses and sw/Wisconsin/H04YS2/04 (sw/WI/04) were included in *in vitro* immunological assays. The latter virus is a North American triple-reassortant H1N1 SIV, which has all the internal genes except M very similar to those of A/IN/11, and it was therefore used to estimate immune responses against the internal proteins of the H3N2v. Viruses used for pig inoculation were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs (sw/Gent/08) or in Madin-Darby canine kidney (MDCK) cells (A/IN/11) for ≤3 passages. Virus stocks used in immunological assays underwent one additional passage in eggs or MDCK cells.

4.1.3.2 Inoculation and challenge of pigs

Thirty-five 5-week-old pigs were obtained from a conventional farm and were randomly assigned to three groups. All those pigs were confirmed to be seronegative to all endemic European SIVs by HI and VN assays prior to the experiment. Each group was housed in a separate biosafety level-2 HEPA-filtered isolation unit. After acclimation for 1 week, two groups were inoculated with sw/Gent/08 (n=12) or A/IN/11 (n=11), respectively. One group was left uninoculated and served as the challenge control group (n=12). Eight weeks later, all groups were challenged with A/IN/11. All inoculations were performed intranasally with 7.0 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) influenza virus in 3 ml (1.5 ml per nostril) as described elsewhere. In each group, pigs were euthanized before the inoculation (n=4), and at 3 and 7 dpc (n=3 or 4).

4.1.3.3 Collection of samples

To determine virus excretion, nasal swabs were collected daily from all pigs from 0-7 days post-primary inoculation (dpi) and from 0-7 days post-challenge (dpc), or until euthanasia. Blood samples for serological examinations were collected from all pigs prior to the first inoculation and challenge. To determine virus replication in the respiratory tract, five samples (nasal mucosa olfactory part, tonsil, and trachea, the apical, cardiac, and diaphragmatic lobes of the left and right lung, and accessory lung) were collected separately for virus titration at necropsy. Gross lung lesions were scored by visual inspection. At necropsy, additional nasal swabs and BALF were collected to determine mucosal antibody titers to influenza viruses. BALF was obtained by lavaging the right lung with 200 ml of phosphate-buffered saline (PBS). Also, nasal mucosa respiratory part, TBLNs, and blood were collected for isolation of mononuclear cells (MNCs).

4.1.3.4 Virus titration

Cotton swabs were weighed before and after collection to determine virus titers per 100 mg nasal secretions. Swabs from both nostrils were suspended in 1 ml sterile PBS supplemented with antibiotics. Tissues were weighed and homogenized in sterile PBS with antibiotics to obtain 20% (w/v) homogenates. Nasal swab specimens and tissue homogenates were titrated in MDCK cells as described elsewhere (Van

Reeth *et al.*, 2003). Virus titers were expressed as $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) per 100 milligram (nasal swabs) or per gram (tissues).

4.1.3.5 Serological assays

Serum antibody responses were examined in HI, VN and NI assays, as described elsewhere (Leuwerke *et al.*, 2008; Sandbulte *et al.*, 2009; Van Reeth *et al.*, 2003). All sera were examined against sw/Gent/08, A/IN/11, and sw/WI/04 in all three assays. Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete inhibition of HA of 4 hemagglutinating units (HAU) of virus (HI assay), 50% neutralization of 100 $TCID_{50}$ of virus in MDCK cells (VN assay), or 50% reduction of NA activity (NI assay). Nasal swabs and BALF collected at euthanasia were tested in VN assays against the challenge virus A/IN/11 only. Starting dilutions were 1:2 in the VN assay, and 1:10 in the HI and NI assays.

4.1.3.6 Isolation of MNCs

To obtain MNCs for analysis from the nasal mucosa, mucosal tissues were carefully peeled from the medial side of the ventral turbinates and from the nasal septum as described elsewhere (Larsen *et al.*, 2000). Tissues were physically disrupted and digested with 1 mg/ml collagenase IV (Invitrogen) and 0.01 mg/ml DNase I (Roche) in RPMI-1640 (Gibco) supplemented with 5% fetal calf serum, 100 mg/ml of gentamicin, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C for 3-4 h. The supernatant of this digestion was filtered through a series of cell strainers at 70- and 40-µm and washed twice with Dulbecco's Phosphate-Buffered Saline (Solarbio). The cell suspension was centrifuged over a discontinuous Percoll (GE Healthcare) density gradient (1.080 g/ml and 1.055 g/ml). TBLNs were mechanically disrupted through a 60-mesh tissue smashing screen (Sigma). The cell suspensions and peripheral blood were centrifuged over Ficoll-Paque Plus gradient (GE Healthcare, 1.078 g/ml). Following Percoll or Ficoll-Paque Plus purification, cells at the interface were collected and washed twice in DPBS. Those purified MNCs were suspended in complete RPMI (RPMI 1640 enriched with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mg/ml of gentamicin, 100 U/ml penicillin and 100 mg/ml streptomycin). The viability of each MNC preparation was assessed by trypan blue staining and >95% of the cells were confirmed to be viable.

4.1.3.7 ELISpot assays for antibody secreting cells (ASCs)

An ELISpot assay for IgG and IgA ASCs was performed as previously described (Kitikoon *et al.*, 2014) with slight modifications. Briefly, 96-well membrane plates (MAIPS4510, Millipore) were prewetted with 70% ethanol, washed, and coated overnight at 4°C with 200 HAU of live purified influenza virus. Wells coated with purified sham MDCK medium served as negative controls. The next day, the plate was washed and blocked with complete RPMI for 2 h at 37°C. The blocking medium was removed, wells were incubated at 37°C and 5% CO_2 for 18 h with 5×10^5, 5×10^4, and 5×10^3 MNCs (each dilution in duplicate). After incubation, plates were washed and incubated with anti-porcine IgG or IgA monoclonal antibody for 2 h at 37°C. Then, plates were washed and incubated with 0.5 µg/ml horseradish peroxidase-labeled (HRP) goat anti-mouse immunoglobulins (DAKO) for 1 h at 37°C. After washing, spots were developed using TMB Substrate System (Sigma). Plates were scanned and spots were enumerated using CTL-ImmunoSpot S5 UV analyzer and ImmunoSpot software.

4.1.3.8 ELISpot assays for ISC

An ELISpot assay for ISC was performed as previously described (Brockmeier *et al.*, 2012; Gorres *et al.*, 2011) with slight modifications. Briefly, 96-well membrane plates (MAIPS4510, Millipore) were prewetted with 70% ethanol, washed, and coated overnight at 4°C with 6 µg/ml anti-porcine IFN-γ (P2G10; BD Biosciences). The next day, the plate was washed and blocked with complete RPMI for 2 h at 37°C. The blocking medium was removed, and 5×10^5 MNCs were seeded per well. Treatment preparations were added to appropriate wells (each treatment was carried out in duplicate), and the plates were incubated for 18 h at 37°C and 5% CO_2. Treatments included live purified influenza virus at a multiplicity of infection (MOI) of 1, purified sham MDCK medium, or concanavalin A added at 5 µg/ml. After 18 h, plates were washed and incubated with 0.5 µg/ml anti-porcine IFN-γ detection antibody (P2C11; BD Biosciences) for 2 h at 37°C. Then, plates were washed and incubated with 0.5 µg/ml streptavidin-HRP conjugate for 1 h at 37°C (Invitrogen). After washing, spots were developed using TMB Substrate System (Sigma). Plates were scanned and spots were enumerated using CTL-ImmunoSpot S5 UV analyzer and ImmunoSpot software.

4.1.3.9 Statistics

Samples that tested negative for virus were given a numeric value of 1.6 $\log_{10}$ $TCID_{50}$ per 100 milligram (nasal swabs) or per gram (tissues). Samples that tested negative in the serological assays were assigned a value corresponding to half of the minimum detectable titer. Viral shedding of each virus was quantified by calculation of the area under the curve (AUC), which is obtained by plotting viral titers versus each time point of sample collection. Mann-Whitney U tests were used to compare antibody titers, virus titers in respiratory tissues, and numbers of ASC or ISC. $P<0.05$ was considered statistically significant.

4.1.4 Results

4.1.4.1 Genetic relationship between viruses

Percentages of amino acid identity between the HA1, NA, M, and NP of A/IN/11, sw/Gent/08 and WI/04 are summarized in Table 1. The two H3N2 viruses had an amino acid identity of 79.6% in HA1 and 82.5% in NA, with 18 and 20 amino acid differences in the respective H3 and N2 antigenic sites. As expected, the M protein of A/IN/11 was most similar to that of sw/Gent/08 (97.0% homology), whereas the NP protein of A/IN/11 was most related to that of WI/04 (98.6% homology).

Table 1. Comparisons of genes of the H3N2v virus A/IN/11 used for challenge and sw/Gent/08 (H3N2) and sw/WI/04 (H1N1) viruses

	% identity* to A/IN/11 genes			
	HA1	NA	M	NP
sw/Gent/08	79.6	82.5	97.0	93.2
sw/WI/04	40.1	42.0	92.9	98.6

*: % identity at the amino acid level.

4.1.4.2 Virus replication in nasal swabs and respiratory tissues

After the primary inoculation with A/IN/11 or sw/Gent/08, all pigs had high virus titers (up to 6.7 $TCID_{50}$/100 mg) in nasal swabs for 5-6 days. Mean virus titers in nasal swabs of each group are shown in Figure 1. The average AUC was 24.5 for A/IN/11, and 23.7 for sw/Gent/08. The challenge control pigs tested virus-negative at all time points (AUC=0). After challenge with A/IN/11, all challenge controls shed high titers

of virus (up to 7.2 $TCID_{50}$/100 mg) for 5-6 days, with an AUC of 24.1. Virus excretion was undetectable in the A/IN/11-A/IN/11 group (AUC=0) and significantly reduced in the sw/Gent/08-A/IN/11 group in both magnitude and duration (AUC=8.2).

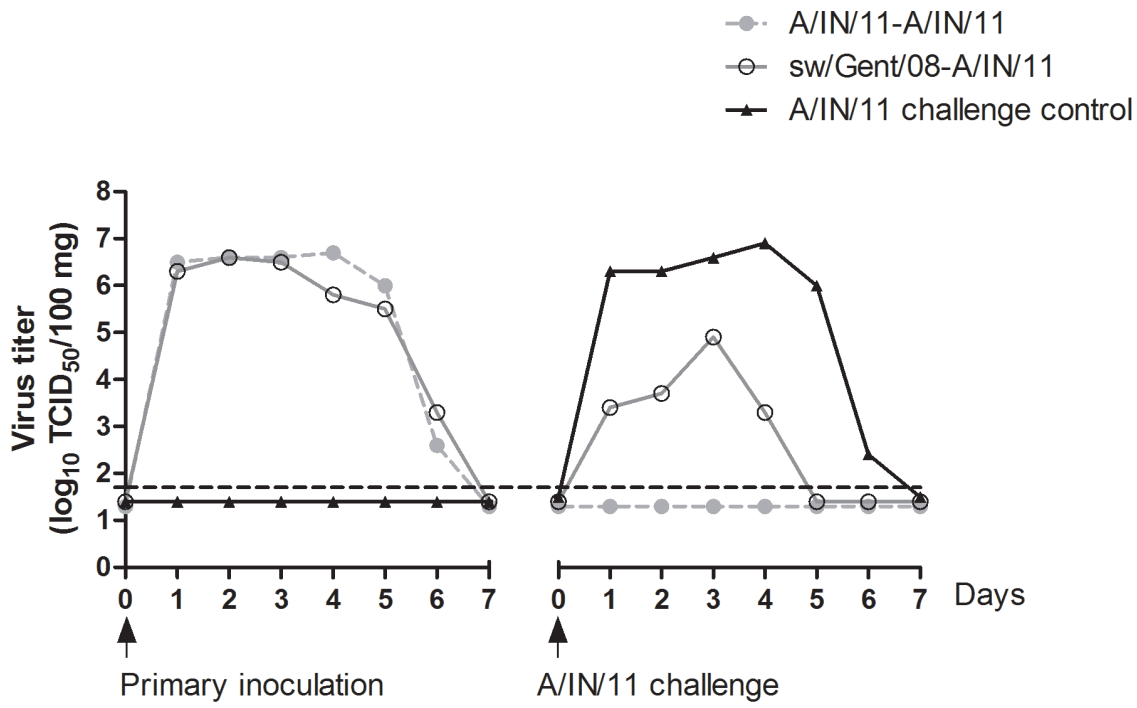

Figure 1. Nasal virus excretion after primary inoculation with sw/Gent/08 or A/IN/11 and after challenge with A/IN/11. Nasal swabs were collected daily from all pigs from 0-7 days post-primary inoculation (dpi) and from 0-7 days post-challenge (dpc), or until euthanasia. Mean virus titers in nasal swabs of each group are given. The horizontal broken line represents the detection limit (1.7 log_{10} $TCID_{50}$/100 mg).

Tissues of the respiratory tract were negative for the challenge virus in all 3 groups at 0 and 7 dpc. At 3 dpc, virus titers were high in all 4 challenge control pigs and undetectable in A/IN/11-A/IN/11 pigs (Figure 2). Virus isolation rates and virus titers in all examined tissues were significantly reduced in sw/Gent/08-A/IN/11 pigs ($p<0.05$).

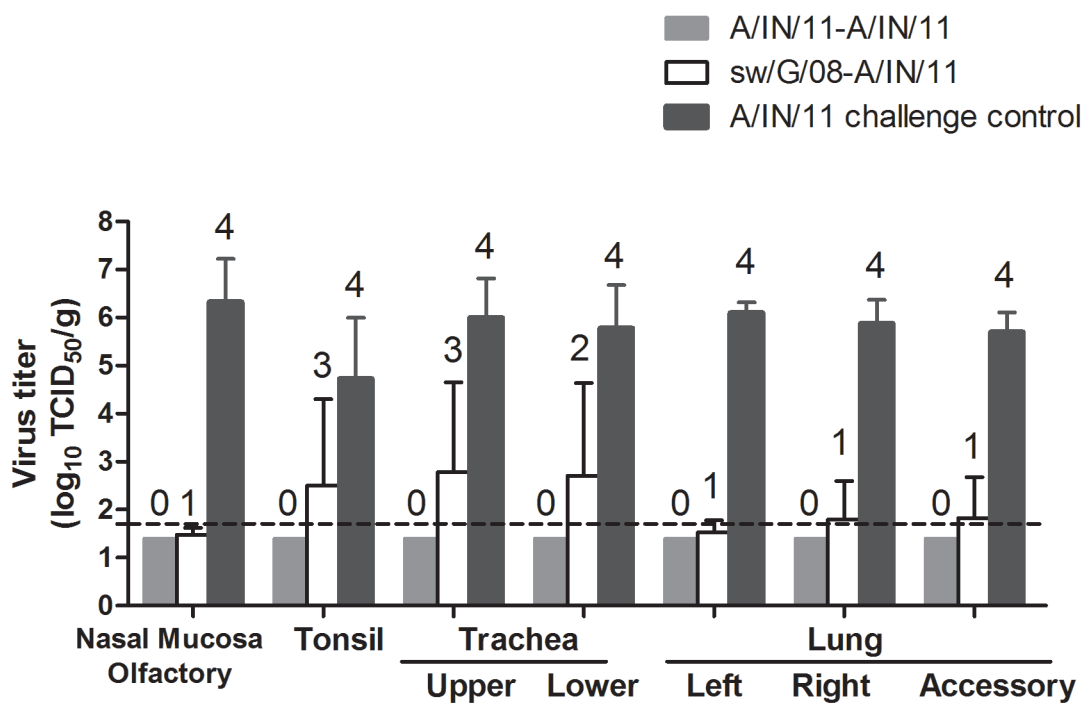

Figure 2. Virus titers in the respiratory tract 3 days after challenge with A/IN/11 in challenge controls and pigs previously infected with A/IN/11 or sw/Gent/08 virus. Four pigs were analyzed in each group and bars represent mean virus titers with standard deviation (SD). Numbers above the bars represent the number of positive pigs in each group. The horizontal broken line represents the detection limit (1.7 $\log_{10}$ $TCID_{50}$/g).

4.1.4.3 Serological profile before and after the primary inoculation

All pigs were seronegative to A/IN/11, sw/Gent/08 and sw/WI/04 in all assays prior to the primary inoculation. Eight weeks after the primary inoculation with A/IN/11 or sw/Gent/08 (at the time of challenge), pigs had the highest antibody titers ($p<0.05$) to the homologous virus in all assays, and geometric mean titers (GMTs) are shown in Table 2. Cross-reactivity between both H3N2 viruses was minimal in the VN assay, and absent in HI and NI assays. The challenge controls remained seronegative to all three influenza viruses.

Table 2. Geometric mean antibody titers at 8 weeks after primary inoculation in hemagglutination-inhibition (HI), virus-neutralization (VN), and neuraminidase-inhibition (NI) assays

		Antibody titer against								
	No. of	A/IN/11			sw/Gent/08			sw/WI/04		
Groups	pigs	HI	VN	NI	HI	VN	NI	HI	VN	NI
A/IN/11-A/IN/11	11	75	466	75	<10	5	<10	<10	<2	<10
sw/Gent/08-A/IN/11	12	<10	7	<10	30	204	202	<10	<2	<10
A/IN/11 challenge control	12	<10	<2	<10	<10	<2	<10	<10	<2	<10

Abbreviations: A/IN/11, A/Indiana/08/11 (H3N2 variant); sw/Gent/08: sw/Gent/172/08 (H3N2 SIV); sw/WI/04: sw/Wisconsin/2004 (H1N1 SIV).

4.1.4.4 VN antibodies to the challenge virus in serum, nasal swabs, and BALF before and after A/IN/11 challenge

At the time of challenge, VN antibodies to the A/IN/11 challenge virus were detected in serum, nasal swabs, and BALF of A/IN/11-A/IN/11 pigs (Figure 3). Minimal antibody titers were found in sw/Gent/08-A/IN/11 pigs, in serum only. After challenge with A/IN/11, VN antibodies to the challenge virus were for the first time detected in challenge controls in serum, nasal swabs and BALF at 7 dpc, while such antibodies remained at pre-challenge level in A/IN/11-A/IN/11 pigs in all tissue samples ($p>0.05$). The sw/Gent/08-A/IN/11 pigs developed or had an increase in VN antibodies to A/IN/11, titers in serum and nasal swabs were higher than those in both other groups ($p<0.05$).

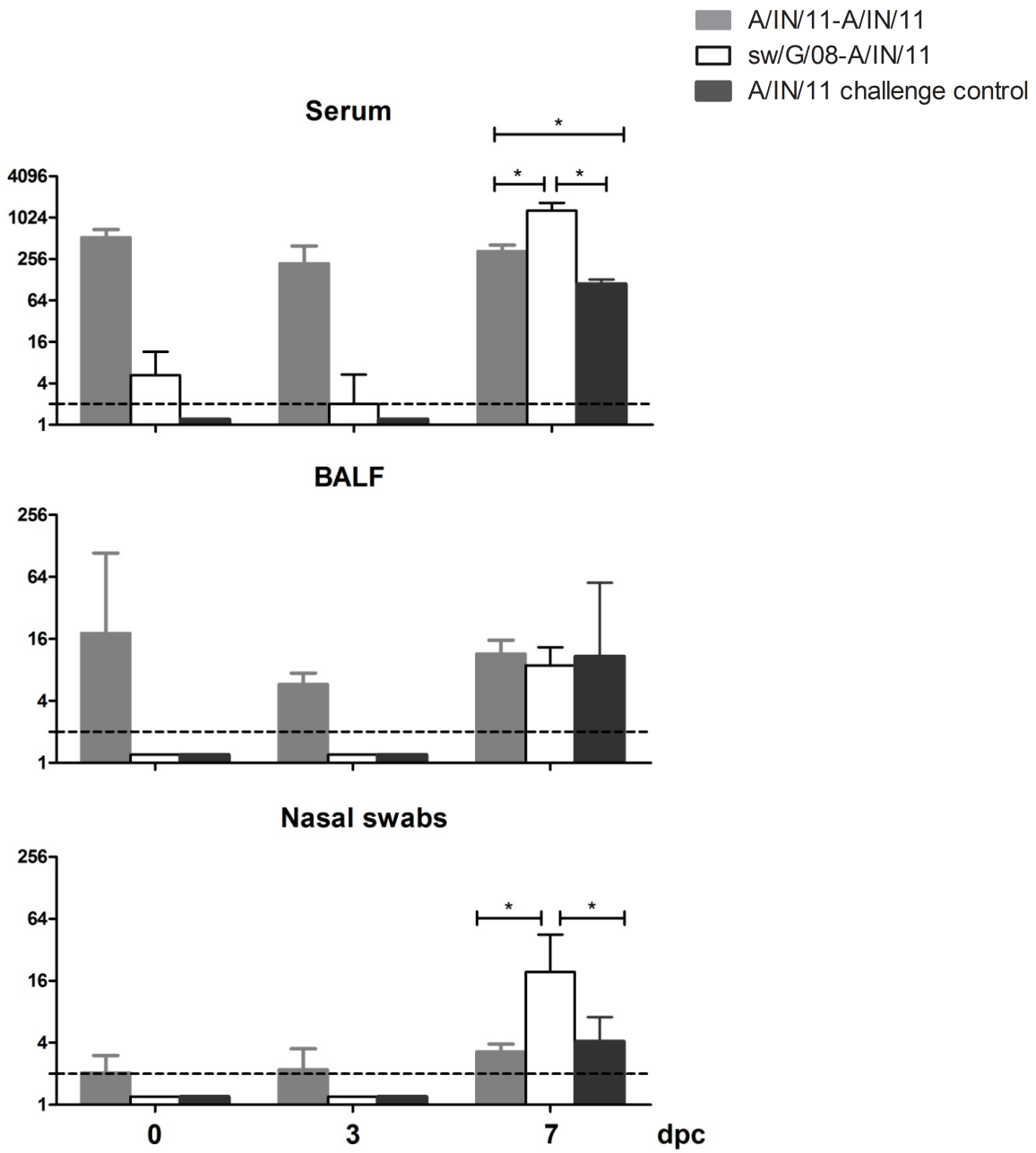

Figure 3. Virus-neutralizing (VN) antibody titers against the challenge virus A/IN/11 in serum, bronchoalveolar lavage fluid (BALF), and nasal swabs in pigs euthanized at 0, 3 and 7 days post challenge. Bars represent geometric mean VN antibody titers with standard deviation (4 pigs per group at each time point, except for the A/IN/11-A/IN/11 group at 7 dpc [n=3]); the horizontal broken line represents the detection limit (2); * $p<0.05$.

4.1.4.5 Influenza virus specific ASC responses

At the time of challenge, all challenge control pigs had undetectable influenza virus-specific IgA and IgG ASC in nasal mucosa, TBLN and blood (Figure 4, 5 and 6). In contrast, pigs inoculated with sw/Gent/08 or A/IN/11 showed IgA and IgG ASC responses against all three viruses, predominantly in nasal mucosa. Cross-reaction was stronger between the two H3N2 viruses than between H3N2 and H1N1 viruses. Responses to IN/11 in respect of IgG ASCs were significantly higher in the A/IN/11- than sw/Gent/08-inoculated group (40 and 4 ASCs per 10^6 MNCs, respectively; $p<0.05$) (Figure 4), whereas IN/11-specific IgA ASC responses were similar in both groups (81 and 23 ASCs per 10^6 MNCs, respectively; $p>0.05$).

After challenge with A/IN/11, IgA and IgG ASC responses to the challenge virus were detected in challenge control pigs at 7 dpc, mainly in peripheral blood (11 IgA and 115 IgG ASC per 10^6 MNCs) and only few in nasal mucosa. This was in contrast with the more substantial ASC responses in nasal mucosa in the other two groups. In nasal mucosa, IgA and IgG ASCs specific to IN/11 showed minimal increases in the A/IN/11-A/IN/11 pigs post-challenge. In contrast, the sw/Gent/08-A/IN/11 group exhibited a 20-fold increase in IgA and a 200-fold increase in IgG ASC responses to A/IN/11 at 7 dpc (476 IgA and 789 IgG ASC per 10^6 MNCs). Meanwhile, IgA and IgG ASC responses to sw/Gent/08 and sw/WI/04 increased to similar levels as those to A/IN/11 ($p>0.05$), which were significantly greater than those in both other groups ($p<0.05$).

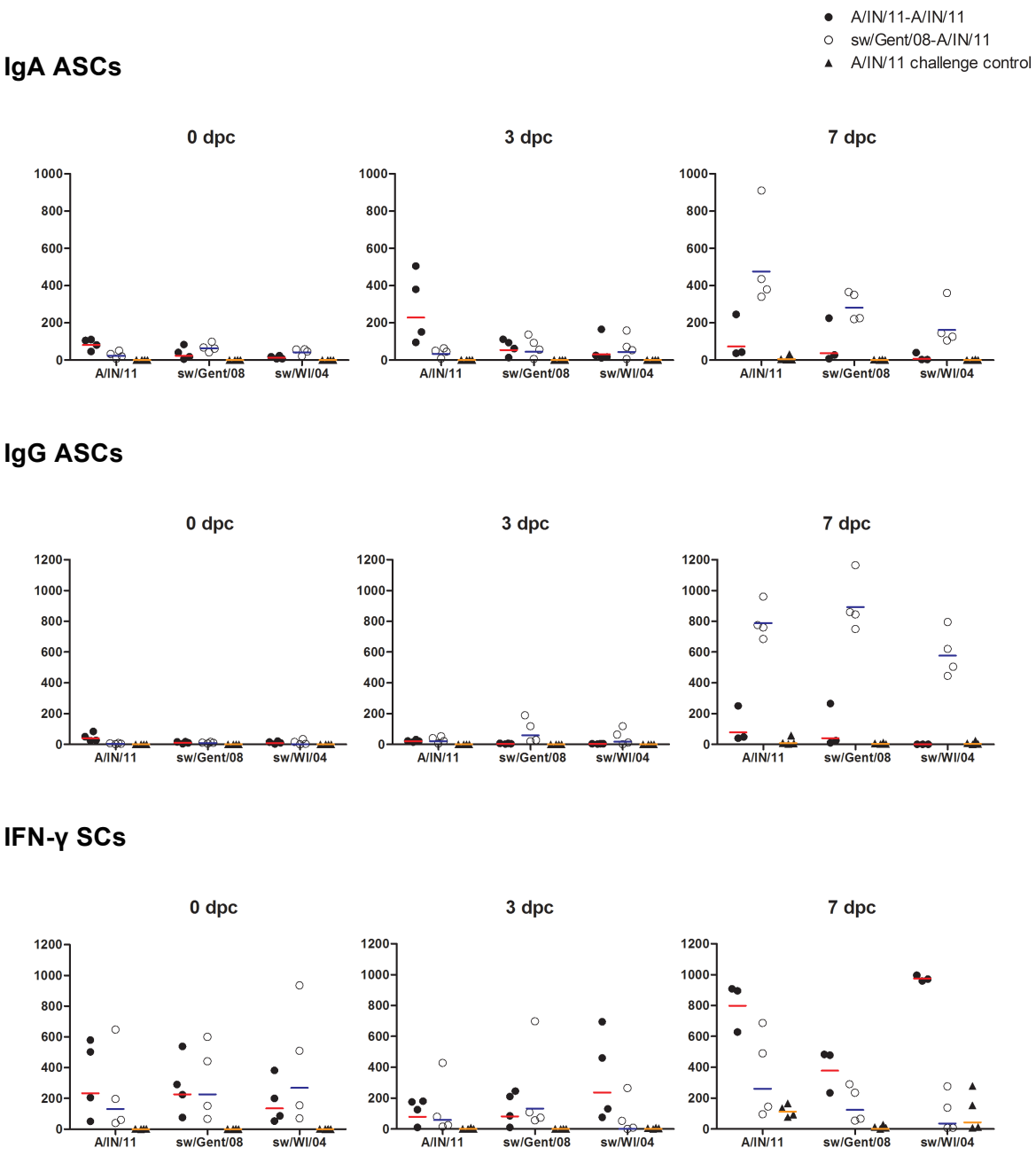

Figure 4. Kinetics of IgA and IgG antibody and IFN-γ secreting cell responses specific to A/IN/11, sw/Gent/08 and sw/WI/04 before and after challenge with A/IN/11 in nasal mucosa (4 pigs per group at each time point, except for the A/IN/11- A/IN/11 group at 7 dpc [n=3]). The dots and triangles represent individual pigs; the horizontal line represents the group geometric mean. ASCs: antibody secreting cells; SC: secreting cells.

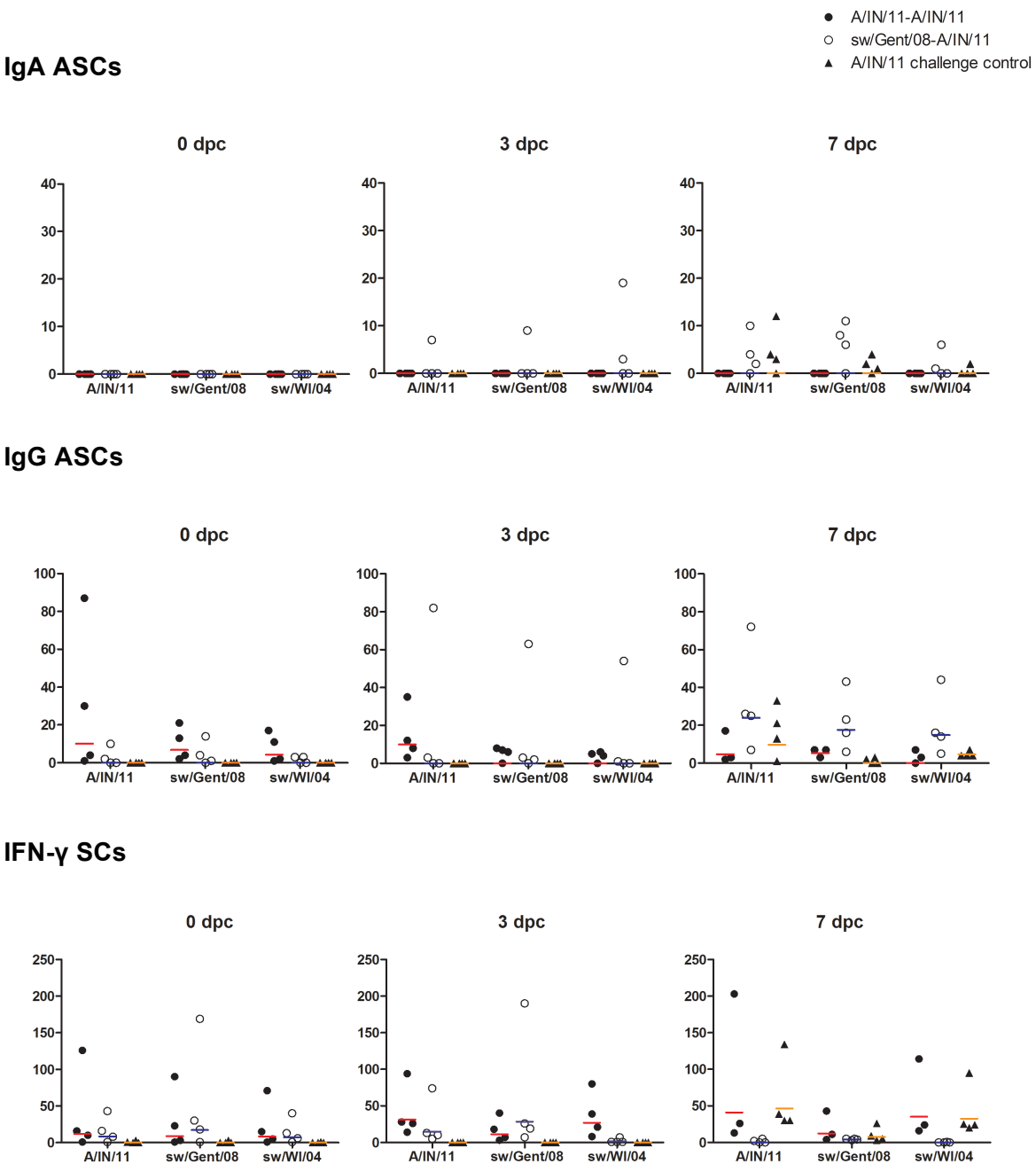

Figure 5. Kinetics of IgA and IgG antibody and IFN-γ secreting cell responses specific to A/IN/11, sw/Gent/08 and sw/WI/04 before and after challenge with A/IN/11 in tracheobronchial lymph nodes (4 pigs per group at each time point, except for the A/IN/11- A/IN/11 group at 7 dpc [n=3]). The dots and triangles represent individual pigs; the horizontal line represents the group geometric mean. ASCs: antibody secreting cells; SC: secreting cells.

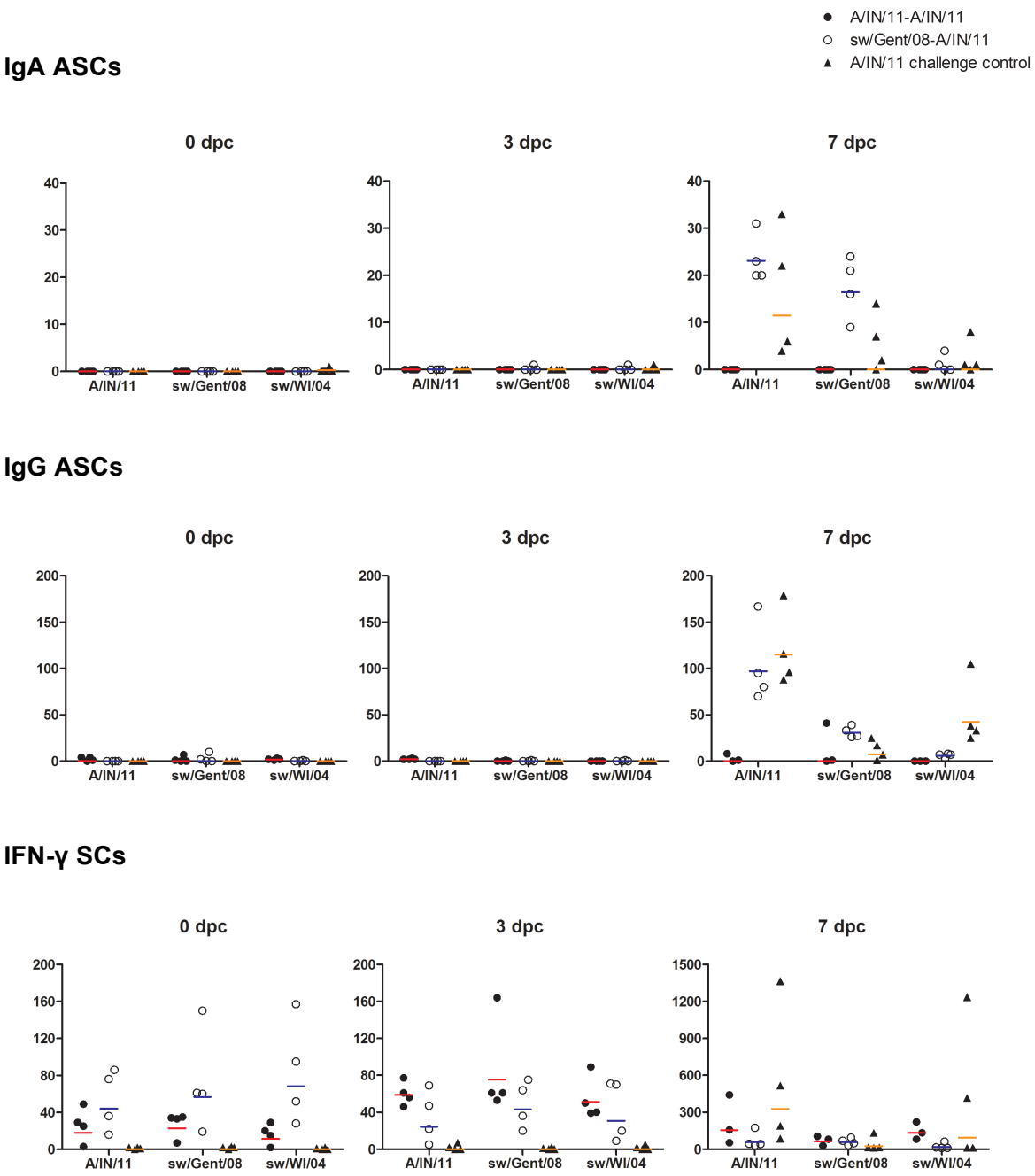

Figure 6. Kinetics of IgA and IgG antibody and IFN-γ secreting cell responses specific to A/IN/11, sw/Gent/08 and sw/WI/04 before and after challenge with A/IN/11 in peripheral blood (4 pigs per group at each time point, except for the A/IN/11- A/IN/11 group at 7 dpc [n=3]). The dots and triangles represent individual pigs; the horizontal line represents the group geometric mean. ASCs: antibody secreting cells; SC: secreting cells.

4.1.4.6 Influenza virus specific IFN-γ responses

At the time of challenge, challenge control pigs had few influenza virus-specific ISCs (≤2 per 10^6 MNCs) in nasal mucosa, TBLN and blood (Figure 4, 5 and 6). In contrast, sw/Gent/08- and A/IN/11-inoculated pigs had ISC responses to all three viruses, which were at similar levels ($p>0.05$) and predominantly in nasal mucosa. Numbers of A/IN/11-specific ISC in nasal mucosa were similar between A/IN/11- and sw/Gent/08-inoculated pigs (234 and 131 ISCs per 10^6 MNCs, respectively; $p>0.05$) (Figure 4).

After challenge with A/IN/11, A/IN/11-specific ISC responses were induced in challenge control pigs in nasal mucosa, TBLN, and most in peripheral blood at 7 dpc (113, 47 and 328 ISCs per 10^6 MNCs, respectively). The A/IN/11-A/IN/11 pigs showed a mild increase in ISCs specific to A/IN/11 at 7 dpc, especially in the nasal mucosa and peripheral blood (799 and 154 ISCs per 10^6 MNCs, respectively). Meanwhile, ISCs to sw/Gent/08 and sw/WI/04 also increased to similar levels as those to A/IN/11 ($p>0.05$). ISC responses to all three viruses remained at pre-challenge level in sw/Gent/08-A/IN/11 pigs ($p>0.05$).

4.1.5 Discussion

Pigs previously infected with a European H3N2 SIV were partially protected against a North American H3N2v, as shown by reduced virus titers in nasal swabs and respiratory tissues post-challenge. Both swine-origin H3N2 viruses have their HA and NA genes originate from historical human seasonal H3N2 viruses from different decades, and they are genetically and antigenically distinct from one another. There was approximately 80% amino acid identity between their HAs and NAs, no detectable cross-reactivity in HI and NI assays and minimal cross-reactivity in the VN assay. A similar significant reduction in the magnitude and duration of nasal virus shedding of A/IN/11 was observed in ferrets pre-infected with a recent human seasonal H3N2 virus A/Perth/16/2009 (Houser *et al.*, 2013). The two viruses had 86% amino acid identity in their HA1 segments, and cross-reactivity was undetectable in HI assays with ferret post-infection sera. Yet, a complete cross-protection can occur between H3N2 viruses with greater genetic relatedness, as between sw/Gent/08 and a historical human seasonal H3N2 virus A/Victoria/3/75 that have their HAs and NAs of the same origin (Qiu *et al.*, 2013). The rapid viral clearance in sw/Gent/08-inoculated pigs after A/IN/11 challenge correlated well with

the higher A/IN/11-specific VN antibody titers in serum and nasal swabs, and the higher A/IN/11-specific IgA and IgG ASC responses in nasal mucosa at 7 dpc than those in challenge controls. The ASC responses appear to target at least in part the viral internal proteins, as the response to H1N1 SIV showed a parallel albeit lower increase after challenge in sw/Gent/08-inoculated pigs. Our results are in agreement with a previous report in which infection with pH1N1 primed for memory B cell responses in human nasal-associated lymphoid tissues, which produce cross-reactive antibodies to human seasonal H1N1 viruses as well as avian H5N1 viruses (Mahallawi *et al.*, 2013). In the present study, ISC responses, which target the conserved epitopes in surface and internal proteins, showed a higher level of cross-reactivity among all three viruses than ASC responses, since similar recall responses were obtained after the primary infection against all three viruses. However, virus-specific ISC responses did not increase after the heterovariant virus challenge. This points towards a more significant role of antibody-mediated immunity and is in line with a study in the mouse model. In the latter study, seasonal H1N1 influenza virus infection induced cross-protective immunity to the pH1N1 virus through a $CD8^+$ T cell-independent, B cell-dependent mechanism (Fang *et al.*, 2012). On the other hand, protection against the homologous A/IN/11 virus in our study was associated with an anamnestic response of virus-specific ISC in the nasal mucosa 7 dpc, suggesting a T cell memory response, whereas an increase in IgA ASC in nasal mucosa 3 dpc suggested a memory response but this could not be confirmed 7 dpc. Nevertheless VN antibodies were present in these pigs at re-infection, so that immune complexes will have been formed. It is well-known that antigen complexed with IgG is much more efficient in inducing $CD4^+$ T cell responses than soluble antigen (de Jong *et al.*, 2006), whereas suppression of antibody responses has been described for particulate antigen, most likely by B cell epitope masking (Hjelm *et al.*, 2006). In a previous study re-infection of pigs with the same homologous SIV also resulted in increased virus-specific ISC responses in nasal mucosa, TBLN and spleen at 14 dpc, but minimal increases in virus-specific IgG and IgA ASC responses (Larsen *et al.*, 2000).

Our study is in agreement with the previous mentioned study that demonstrates the nasal mucosa is the predominant site of both IgG and IgA ASCs after SIV infection (Larsen *et al.*, 2000). The locally produced antibodies may directly contribute to the protection in the upper respiratory tract. Indeed, the sw/Gent/08-A/IN/11 pigs had

highest A/IN/11-specific VN antibody titers in nasal swabs but similar titers in BALF compared to the other two groups at 7 dpc, suggesting A/IN/11-specific VN antibodies in nasal swabs were at least partially locally produced. The highest post-challenge VN antibody titers against A/IN/11 were found in serum, which may be due to the fact that nasal swabs and BALF samples are much more diluted than serum samples during sample collection and processing. In spite of the local immunity in nasal mucosa, the challenge virus was found to replicate mainly in the upper respiratory tract of the sw/Gent/08-A/IN/11 pigs, while it was largely undetectable in the lungs. Although not examined in this study, studies in mice suggest the strong virological protection in the lungs may be associated with influenza virus-specific ASCs located in the lung parenchyma (Baumgarth & Kelso, 1996; Jones & Ada, 1987). In addition, systemic IgG antibodies can much more easily transudate into alveolar epithelia than to the nasal mucosa (Renegar *et al.*, 2004; Rudin *et al.*, 1999), and they may reduce virus replication in the lung. Our study also shows nasal mucosa is the predominant site of ISCs, which does not agree with the previous report that shows ISC responses were detected predominantly in the TBLN and spleen after intranasal inoculation with a H1N1 SIV (Larsen *et al.*, 2000). We did not include spleen for ELISpot analysis, but we found the ISCs in nasal mucosa induced by infection with either sw/Gent/08 or A/IN/11 were almost 20-fold more than those in Larsen's study at 42 dpi.

Our study in pigs is in agreement with the observation in humans that pre-existing infection-immunity can offer significant protection against an antigenically distinct strain within the same subtype, though infection is not completely prevented. As an example, young adults and children with substantial antibody and CMI responses to contemporary seasonal H1N1 viruses in 2000s were still infected by the pH1N1 virus (Skowronski *et al.*, 2011). In contrast, protecting mice against influenza virus is very easy (Dormitzer *et al.*, 2011). Our study suggest pigs may serve as a valuable model to study protective immunity against influenza. Pigs are the natural hosts for influenza viruses and they are readily susceptible to a variety of human influenza A viruses without prior adaption. The pathogenesis of influenza is remarkably similar in pigs and in humans, and there are also striking similarities in their immune responses (Khatri *et al.*, 2010; Van Reeth *et al.*, 2012). Both IgG and IgA antibodies in nasal secretions have been correlated with protection against influenza in humans (Boyce *et al.*, 1999; Clements *et al.*, 1986; Clements *et al.*, 1983). The nasal associated

lymphoid tissues are considered to be important induction sites for both mucosal and systemic immunity to influenza viruses in humans and pigs (Wiley *et al.*, 2001; Zuercher *et al.*, 2002), but it is usually very difficult to obtain those tissues from humans. Unlike ferrets and mice, the large size of respiratory tissues as well as the availability of various immunological reagents allows using pigs to study the mucosal and cellular immune responses against influenza.

In summary, our study has shown prior infection with an H3N2 influenza virus failed to prevent the challenge infection of a genetically and antigenically distinct strain in pigs, but virus replication in both upper and lower respiratory tract was reduced. This heterovariant protection was associated with substantial IgA and IgG ASC boost responses in nasal mucosa. The pig can serve as a valuable model to study human mucosal immune responses and to improve our understanding of broad-spectrum immunity to influenza.

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Chapter 4.2

Cross-protection against European swine influenza viruses in the context of immunity against the 2009 pandemic H1N1 virus: studies in the pig model of influenza

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Conditionally accepted in Veterinary Research

4.2.1 Abstract

Pigs are natural hosts for the same influenza virus subtypes as humans and are a valuable model for cross-protection studies with influenza. In this study we have used the pig model to examine the extent of virological protection between a) the 2009 pandemic H1N1 (pH1N1) virus and three different European H1 swine influenza virus (SIV) lineages, and b) these H1 viruses and a European H3N2 SIV. Pigs were inoculated intranasally with representative strains of each virus lineage with 6- and 17-week intervals between H1 inoculations and between H1 and H3 inoculations, respectively. Virus titers in nasal swabs and/or tissues of the respiratory tract were determined after each inoculation. There was substantial though differing cross-protection between pH1N1 and other H1 viruses, which was directly correlated with the relatedness in the viral hemagglutinin (HA) and neuraminidase (NA) proteins. Cross-protection against H3N2 was almost complete in pigs with immunity to H1N2, but was weak in H1N1/pH1N1-immune pigs. In conclusion, post-infection immunity may offer substantial cross-lineage protection against viruses of the same HA and/or NA subtype. True heterosubtypic protection, in contrast, appears to be minimal in natural influenza virus hosts. We discuss our findings in the light of the zoonotic and pandemic risks of SIVs.

4.2.2 Introduction

Swine influenza viruses (SIVs) are important for the swine industry and as zoonotic agents. Moreover, they can lead to the emergence of novel pandemic influenza viruses for humans. In Europe, four lineages of SIV are enzootic in swine populations. An H1N1 virus of wholly avian origin became established in European swine in 1979 (Pensaert *et al.*, 1981). In the mid 1980s, this H1N1 virus reassorted with descendants of the 1968 Hong Kong human pandemic H3N2 virus (Campitelli *et al.*, 1997; Castrucci *et al.*, 1993). The resulting H3N2 SIV lineage has human-like hemagglutinin (HA) and neuraminidase (NA) genes and avian-like internal genes. The third lineage, H1N2, was first reported in 1994, and is a reassortant virus that retained most of the genotype of the H3N2 viruses, but has acquired an H1 gene from human seasonal viruses from the 1980s (Brown *et al.*, 1998; Van Reeth *et al.*, 2000). The 2009 pandemic H1N1 (pH1N1) virus is a reassortant with the NA and matrix (M) genes derived from the European avian-like H1N1 SIV and the remaining genes from North American triple-reassortant H1 SIVs (Garten *et al.*, 2009). The

pH1N1 virus was first detected in humans in April 2009 and only later in swine, but it has become widespread in swine worldwide due to large-scale reverse zoonotic transmissions (Nelson & Vincent, 2015). Thus, while all four SIV lineages have a distinct HA and/or NA, the pH1N1 also has a different set of internal genes compared to the three previously established SIVs. A growing number of reassortants between these four lineages has been reported in recent years, especially between pH1N1 and previously established SIVs (Simon *et al.*, 2014).

The increasing number of H1 SIV lineages in Europe and other continents, and the geographic differences in the prevailing lineages have spurred interest in the extent of cross-protection between them. Prior infection with a European avian-like H1N1 SIV largely protects against subsequent infection with the pH1N1 (Busquets *et al.*, 2010), or with a North American triple-reassortant H1N1 SIV (De Vleeschauwer *et al.*, 2011), despite the absence of cross-reactive serum hemagglutination-inhibition (HI) antibodies against the challenge virus. It remains unknown to what extent prior infection with pH1N1 offers protection against the previously established European H1 SIVs. This question is also of public health concern as the global spread of pH1N1 may generate cross-reactive immunity to some H1 SIVs in the human population, making them less likely candidates for future pandemics.

Apart from cross-protection between variants of the same HA subtype, cross-protection between viruses of different HA subtypes (heterosubtypic protection) has also been described. Heterosubtypic protection has been repeatedly shown in rodents and ferrets (Kreijtz *et al.*, 2007; Laurie *et al.*, 2010; Liang *et al.*, 1994; Straight *et al.*, 2006; Yetter *et al.*, 1980), but only rarely in natural hosts of influenza. In an experimental pig infection study with European SIVs, only one out of five H1N1-immune pigs tested positive for the H3N2 challenge virus in oropharyngeal swabs, for 1 day only. However, challenge control pigs in that study also had minimal virus titers in oropharyngeal swabs, and nasal swabs or tissues of the respiratory tract were not examined (Heinen *et al.*, 2001). Epidemiological data support the existence of heterosubtypic immunity in humans that were exposed simultaneously or consecutively to epidemic human seasonal H1N1 and H3N2 viruses (Lavenu *et al.*, 2004; Sonoguchi *et al.*, 1985). Also, the 1957 pandemic H2N2 virus appeared to have a lower disease incidence in adults previously infected with an H1N1 virus (Epstein, 2006). Yet, the significance and importance of heterosubtypic immunity in natural influenza virus hosts remain unclear. In this study, we sought to study cross-

protection between a) pH1N1 and various H1 SIVs, and b) these distinct H1 SIVs and H3N2. We use the pig as a natural host for SIVs and a model for influenza in humans.

4.2.3 Material and methods

4.2.3.1 Viruses and their genetic and antigenic relationships

Viruses for pig inoculation were propagated in embryonated chicken eggs and used at the third or fourth passage. Their genetic constellations are shown in Figure 1. A/California/04/09 is a representative pH1N1, while sw/Gent/28/10 (H1N1), sw/Gent/26/12 (H1N2) and sw/Gent/172/08 (H3N2) are representative for SIVs that are enzootic in Western Europe. Sw/Cotes d'Armor/0046/08 is an occasionally reported reassortant H1N1 (rH1N1) SIV with the H1 derived from the European H1N2 SIV lineage and the N1 from the European H1N1 lineage (Kyriakis *et al.*, 2011). The sequences of the HA1 and NA segments of the 5 viruses were available in GenBank (accession numbers FN646093, FN646099, KC142127, KC142128 and KP406524-KP406529). The HA1 and NA segments were compared at the amino acid level using the MEGALIGN program (DNASTAR, Madison, WI, USA). Amino acid differences at putative antigenic sites of the H1 and N2 proteins, as defined previously (Brownlee & Fodor, 2001; Colman *et al.*, 1983), were identified by alignment using MEGA5 software (Brownlee & Fodor, 2001; Colman *et al.*, 1983). Antigenic characterization of the 5 viruses was performed by HI, virus-neutralization (VN) and neuraminidase-inhibition (NI) assays, using pig sera collected at 2 weeks after inoculation with each individual virus.

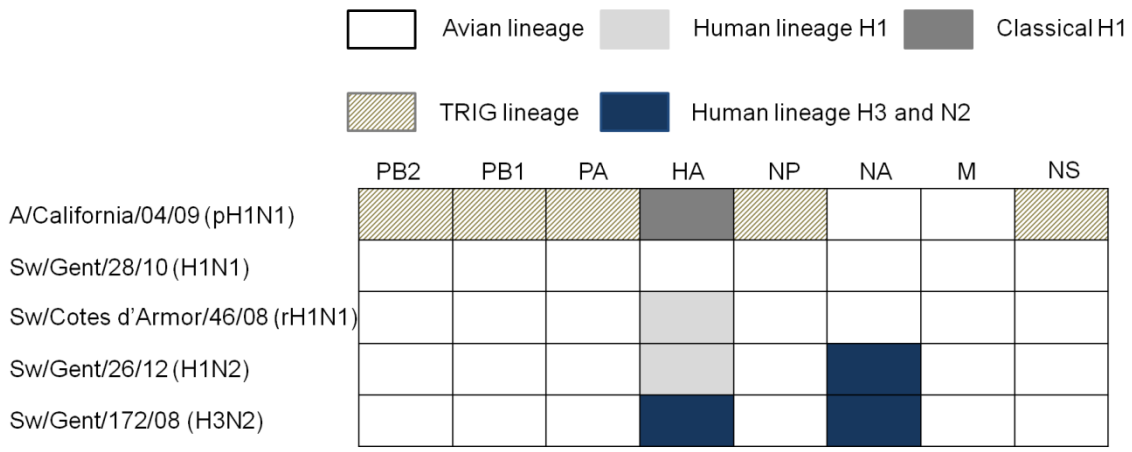

Figure 1. Genetic constellations of the 5 viruses used in this study. Abbreviations: PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural; TRIG, triple-reassortant internal genes, which derived from swine (M, NS and NP), human (PB1) and avian (PB2 and PA) influenza viruses forming a constellation of genes that is well conserved in North American swine influenza viruses.

4.2.3.2 Experimental design

Forty 6-week-old pigs from an influenza negative farm were randomly assigned to 8 groups (n=5) as shown in Table 1. Each group was housed in a separate biosafety level-2 HEPA-filtered isolation unit. All experiments were authorized by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine, Ghent University. Virus inoculations of pigs were performed intranasally, using 7.0 $\log_{10}$ 50% egg infectious doses (EID_{50}) of the respective viruses in 3 ml (1.5 ml per nostril). Pigs were unanesthetized and held in a vertical position with the neck stretched. The inoculum was instilled into the middle nasal cavity by insertion of a 15-mm plastic cannula attached to a 5-ml syringe.

The first experiment was designed to examine whether infection-immunity to pH1N1 offers protection against infection with European H1 SIVs (Table 1). Four groups of pigs (A, B, C and D) were inoculated with pH1N1 virus, and the remaining four groups (E, F, G and H) were mock-inoculated with phosphate-buffered saline (PBS). Six weeks later, the pH1N1-immune pigs were challenged with the same pH1N1 virus (group A), or with one of three European H1 SIVs: H1N1 (group B), rH1N1 (group C), or H1N2 (group D). Three groups of influenza naïve pigs served as H1N1

(group E), rH1N1 (group F), and H1N2 (group G) challenge control groups. Group H was inoculated again with PBS. To determine virus excretion, nasal swabs for virus titration were collected daily from all pigs from 0-8 days post-primary inoculation (dpi) and 0-7 days post-challenge (dpc). Blood samples for serology were collected at 0 and 14 dpi, and at 0, 5, 7, 10 and 14 dpc.

The second experiment aimed at studying the heterosubtypic protection between H1 and H3 viruses (Table 1). Six groups from the first experiment (A, B, D, E, G and H) were challenged with H3N2, 17 weeks after the previous virus inoculation. Nasal swabs for virus titration were collected daily from all pigs from 0-7 dpc, or until euthanasia. Two pigs per group were euthanized at 4 dpc to examine virus titers of the entire respiratory tract: nasal mucosa respiratory and olfactory regions, tonsil, trachea, apical, cardiac, and diaphragmatic lobes of the left and right lungs, and the accessory lung lobe. Each tissue sample was collected and titrated separately. Blood samples for serology were collected at 0 and 14 dpc.

Table 1. Experimental design

	Viruses used for inoculation		
	Experiment 1		Experiment 2
Group	7 weeks[a]	13 weeks[a]	30 weeks[a]
A	pH1N1	pH1N1	H3N2
B	pH1N1	H1N1	H3N2
C	pH1N1	rH1N1	-
D	pH1N1	H1N2	H3N2
E	PBS	H1N1	H3N2
F	PBS	rH1N1	-
G	PBS	H1N2	H3N2
H	PBS	PBS	H3N2

[a] The age of pigs at the time of virus inoculation.

4.2.3.3 Virus titration

Sterile nasal swabs (Copan 160C, Copan Italia S.p.A.) were weighed before and after collection to determine virus titers per 100 mg nasal secretions. Swabs from both nostrils were suspended in 1 ml sterile PBS supplemented with antibiotics. Tissues were weighed and homogenized in sterile PBS with antibiotics to obtain 20% (w/v) homogenates. Nasal swab specimens and tissue homogenates were titrated in

Madin-Darby canine kidney (MDCK) cells by the 50% tissue culture infectious doses ($TCID_{50}$) assay as described elsewhere (Qiu *et al.*, 2013).

4.2.3.4 Serological assays

Serum antibody responses were examined in HI, VN and NI assays, as described elsewhere (Sandbulte *et al.*, 2009; Van Reeth *et al.*, 2003). All sera collected before and at 2 weeks after each inoculation were examined against all 5 viruses in all 3 assays. At 5, 7 and 10 days after challenge with H1 viruses, additional VN assays against the respective challenge viruses were performed. Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete inhibition of HA of 4 hemagglutinating units of virus (HI assay), 50% neutralization of 100 $TCID_{50}$ of virus in MDCK cells (VN assay), or 50% reduction of NA activity (NI assay). Starting dilutions were 1:2 in the VN assay, and 1:10 in HI and NI assays.

4.2.3.5 Statistics

Nasal virus shedding in each group was quantified by calculation of the area under the curve (AUC), which is obtained by plotting viral titers versus each time point of sample collection. Mann-Whitney tests were used to compare antibody levels between any two experimental groups, and before and after inoculation in each group. Differences were considered significant when $p < 0.05$. GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses.

4.2.4 Results

4.2.4.1 Genetic and antigenic relationships between pH1N1 and European SIVs

Genetic and antigenic relationships between pH1N1 and European SIVs

Genetic relationships between viruses were assessed by comparison of the percent amino acid homology in the HA1 and NA sequences (Table 2). Antigenic relationships between viruses were examined in cross-HI, VN and NI assays, using monospecific pig sera (Table 3). The rH1N1 and H1N2 viruses showed 90% amino acid sequence identity in their HA1 and cross-reactivity in HI and VN assays, reflecting the same human-like HA lineages of the two viruses. The HA1 of H1N1, rH1N1 and H1N2 had a similar homology (71-73%) to the classical H1 of pH1N1. Alignment of HA1 antigenic sites of pH1N1 with those of H1N1, rH1N1 and H1N2

revealed 17, 26 and 27 amino acid differences, respectively. Cross-reactivity between pH1N1 and other European H1 SIVs was absent in the HI assay, and rare in the VN assay. H3N2 failed to cross-react with any H1 viruses in HI and VN assays. The H1N1 and rH1N1 showed 97% amino acid identity in their NA, and were closely related to the NA of pH1N1 (91% identity), reflecting the same avian-like N1 lineage of the three viruses. The human-like NAs of H1N2 and H3N2 had 84% amino acid identity and 10 residue differences in antigenic sites. Consistent with the genetic relatedness, cross-reactivity in the NI assay was observed between viruses with the same avian-like N1 or human-like N2 lineage.

Table 2. Percent identity of the amino acid sequences of viral hemagglutinin 1 (HA1) and neuraminidase (NA) genes

	A/California/04/09		Sw/Gent/28/10		Sw/Cotes d'Armor/0046/08		Sw/Gent/26/12		Sw/Gent/172/08	
	HA	NA	HA	NA	HA	NA	HA	NA	HA	NA
A/California/04/09 (pH1N1)	100	100								
Sw/Gent/28/10 (H1N1)	73	91	100	100						
Sw/Cotes d'Armor/0046/08 (rH1N1)	72	91	70	97	100	100				
Sw/Gent/26/12 (H1N2)	71	41	69	40	90	40	100	100		
Sw/Gent/172/08 (H3N2)	34	42	35	40	32	39	33	84	100	100

4.2.4.2 protection against challenge with various European H1 SIVs in pigs with infection-immunity to pH1N1

After primary inoculation with pH1N1, pigs from groups A, B, C and D had similar mean AUC values (range from 24.9-27.4) and nasal shedding during 6-7 days. The mock-inoculated pigs tested negative for virus at all time points.

Figure 2A shows mean virus titers in nasal swabs after challenge with pH1N1, H1N1, rH1N1 or H1N2. The respective challenge control groups (E, F and G) excreted high titers of the challenge viruses for 5-6 days (mean AUC=23.7, 25.1 and 23.6, respectively). In contrast, pH1N1-immune pigs showed complete protection (AUC=0) against challenge with the homologous virus (group A) or with H1N1 (group B), and a almost complete protection against challenge with rH1N1 (group C) (mean AUC=0.1). Virus excretion was detectable in three out of five pigs, for one day only and at minimal virus titers. A slightly weaker protection was observed after challenge with H1N2 (group D): four out of five pigs had virus shedding for 1-3 days (mean AUC=2.1).

Prior to the start of the experiment, pigs were seronegative against all tested influenza viruses in HI, VN and NI assays. At 2 weeks after primary inoculation with pH1N1, pigs from groups A, B, C and D had similar antibody titers ($p > 0.05$) to the homologous virus in all assays. The geometric mean titers (GMTs) are shown in Table 3. At 6 weeks post-inoculation (time of challenge with various H1 viruses), the HI, VN and NI GMTs to pH1N1 were 39, 106 and 260, respectively. Cross-reactive antibodies against H1N1, rH1N1 and H1N2 were undetectable in all pH1N1-immune pigs in the HI assay, but most pigs had low cross-reactive VN titers. Higher cross-reactive NI titers were detected to H1N1 (GMT 36) than to rH1N1 (GMT 17) ($p < 0.05$). The challenge control pigs (groups E, F and G) were still seronegative at the time of challenge with various H1 viruses, but had developed HI, VN and NI antibodies against the respective challenge virus at 14 days post challenge (Table 3). Figure 2B illustrates the more rapid development of VN antibodies against the challenge virus in challenge control pigs than in pH1N1-immune pigs. Anti-pH1N1 antibody titers remained at pre-challenge levels in group A, B and C in all assays ($p > 0.05$), but increased significantly in group D in HI and VN assays ($p < 0.05$).

Table 3. Geometric mean antibody titers in hemagglutination-inhibition (HI), virus-neutralization (VN), and neuraminidase-inhibition (NI) assays at 14 days post-inoculation of pigs with various influenza viruses

		Antibody titer against														
		A/California/04/09			Sw/Gent/28/10			Sw/Cotes d'Armor/0046/08			Sw/Gent/26/12			Sw/Gent/172/08		
Virus for inoculation	No. of pigs	HI	VN	NI	HI	VN	NI	HI	VN	NI	HI	VN	NI	HI	VN	NI
A/California/04/09 (pH1N1)	20	89	76	109	<10	3	16	<10	9	22	<10	4	<10	<10	<2	<10
Sw/Gent/28/10 (H1N1)	5	<10	3	16	80	384	320	<10	3	40	<10	4	<10	<10	<2	<10
Sw/Cotes d'Armor/0046/08 (rH1N1)	5	<10	2	30	<10	<2	40	70	795	211	20	262	<10	<10	<2	<10
Sw/Gent/26/12 (H1N2)	5	<10	<2	<10	<10	<2	<10	15	114	<10	127	1276	46	<10	<2	15
Sw/Gent/172/08 (H3N2)	3	<10	<2	<10	<10	<2	<10	<10	<2	<10	<10	<2	12	160	347	381

Titers against the homologous virus are underlined.

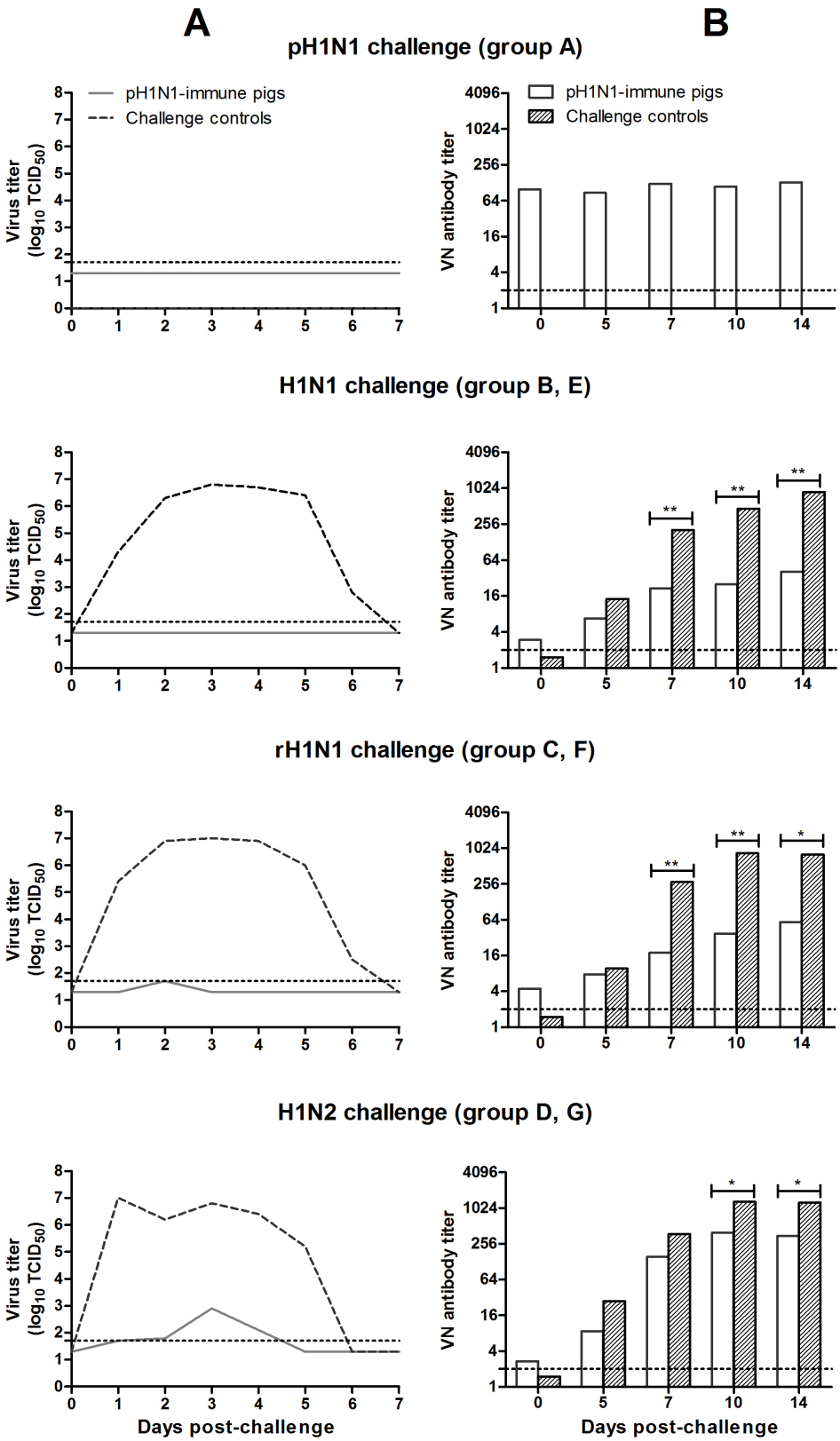

Figure 2. Virus titers in nasal swabs (A) and virus-neutralizing (VN) antibody titers in serum (B) after H1 virus challenge. Horizontal dotted lines represent the detection limit of the assay: 1.7 $\log_{10}$ $TCID_{50}$ for virus titration, 2 for the VN assay. * $p < 0.05$ and ** $p < 0.01$, by the Mann-Whitney test.

4.2.4.3 protection against challenge with a European H3N2 SIV in pigs with infection-immunity to various H1 virus(es)

Figure 3A shows mean virus titers in nasal swabs after challenge with a European H3N2 SIV. All challenge control pigs (group H), pigs immune to pH1N1 (group A) or pH1N1 followed by H1N1 (group B) shed high titers of virus for 4-6 days (mean AUC=23.2, 18.9 or 16.4, respectively). All H1N1-immune pigs (group E) also shed viruses for at least 2-6 days, but the virus titers were reduced (mean AUC=12.3). Only one pig with infection-immunity to H1N2 (group G) and two pigs with infection-immunity to both pH1N1 and H1N2 (group D) had detectable virus excretion (mean AUC=0.14 and 1.65, respectively). Figure 3B shows individual virus titers in the respiratory tract of two pigs of each group at 4 days post H3N2 challenge. Challenge control pigs (group H) were virus-positive in all tissues, except for the olfactory region of the nasal mucosa of one pig. Virus isolation rates and virus titers in the other groups reflected those in nasal swabs. Pigs immune to pH1N1 (group A) or both pH1N1 and H1N1 (group B) showed only a minimal reduction of virus replication. A higher reduction of virus titers was observed in the pigs only immune to H1N1 (group E), while those immune to H1N2 alone (group G) or pH1N1 followed by H1N2 (group D) were almost completely protected against H3N2 replication.

All pigs lacked HI and VN antibodies against H3N2 before the H3N2 challenge. Cross-NI antibodies against H3N2 were only detected in pigs previously exposed to H1N2, and titers were higher in group G (GMT 70) than in group D (GMT 17) ($p < 0.05$). At 14 days after H3N2 challenge, all pigs developed antibodies or showed an increase in pre-existing antibody titers against H3N2 (Table 4). Antibody titers against H1 viruses did not change in all assays after H3N2 challenge ($p > 0.05$).

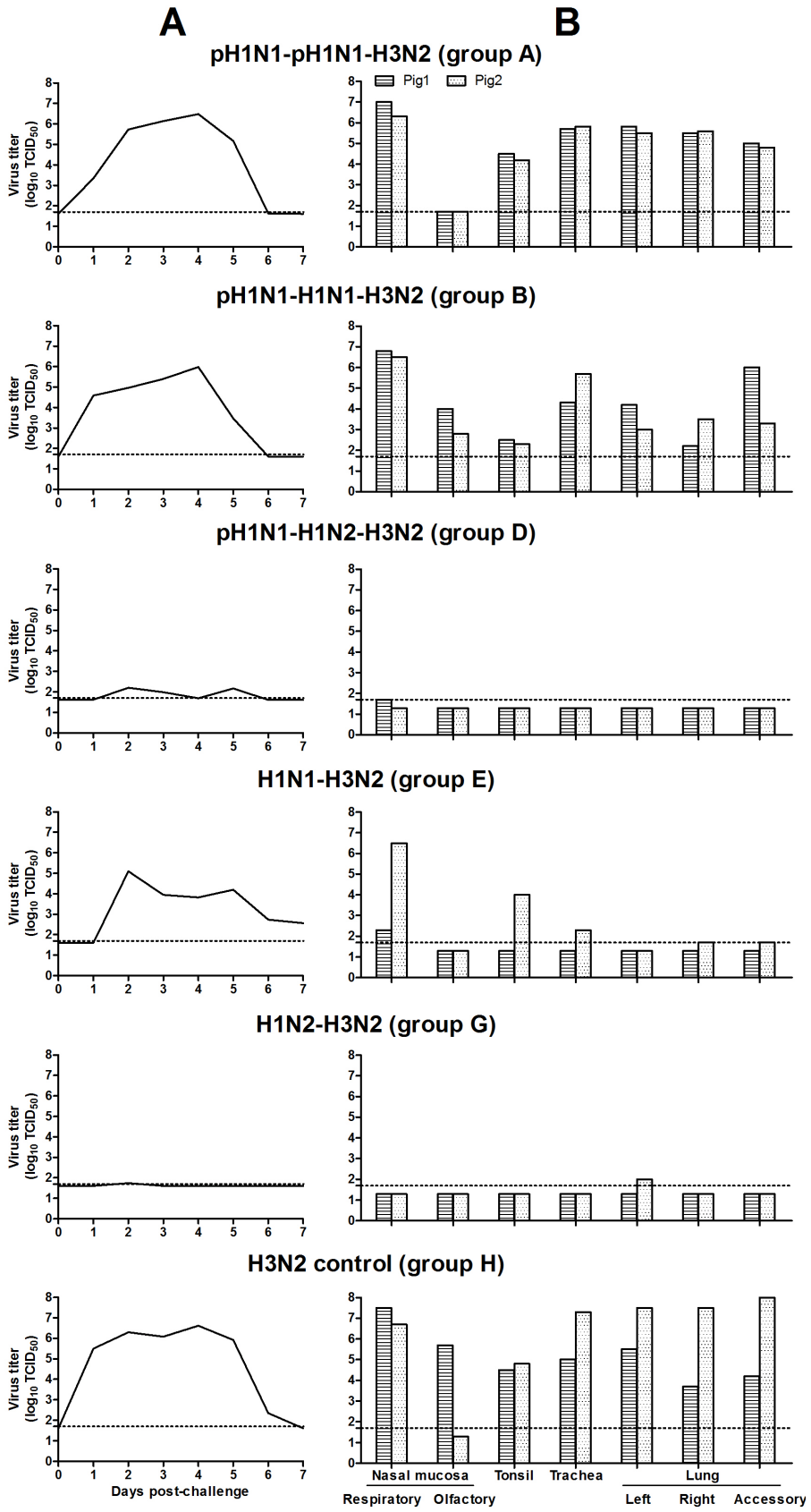

Figure 3. Virus titers in nasal swabs (A) and respiratory tissues (B) after H3N2 challenge. Horizontal dotted lines represent the detection limit for virus titration: 1.7 $\log_{10}$ $TCID_{50}$.

Table 4. Geometric mean antibody titers against the H3N2 virus in hemagglutination- inhibition (HI), virus-neutralization (VN), and neuraminidase-inhibition (NI) assays at 14 days after H3N2 challenge

Group	Virus inoculations	HI	VN	NI
A	pH1N1-6w-pH1N1-17w-H3N2	202	116	320
B	pH1N1-6w-H1N1-17w-H3N2	254	266	254
D	pH1N1-6w-H1N2-17w-H3N2	16	26	320
E	Mock-6w-H1N1-17w-H3N2	127	185	320
G	Mock-6w-H1N2-17w-H3N2	20	16	320
H	Mock-6w-mock-17w-H3N2	160	347	381

4.2.5 Discussion

We have shown a nearly complete cross-protection against replication of European H1 SIV lineages in pigs with infection-immunity against pH1N1, but only a weak cross-protection against the H3N2 subtype in pigs with infection-immunity against various H1N1 viruses. In line with previous studies (Busquets *et al.*, 2010; Kyriakis *et al.*, 2010), all three H1 SIVs used in our studies failed to cross-react with pH1N1 in HI assays with post-infection swine sera, and there was minimal cross-reactivity in VN assays. The VN assay is known to be more sensitive than the HI assay, and the former detects does not only detect antibodies that inhibit the attachment of the virus to target cells, but also antibodies that can block the fusion of the viral and endosomal membranes (Brandenburg *et al.*, 2013). Cross-HI assays with high-titered hyperimmune swine sera have also shown low titers of cross-reactivity between pH1N1 and European avian-like H1N1 but not human-like H1N2 SIVs (Kyriakis *et al.*, 2010). The HA1 of the European H1 SIVs had similarly low percentages (69-71%) of amino acid homology to pH1N1. However, a detailed analysis of the antigenic sites revealed fewer amino acid differences between the classical H1 of pH1N1 and the avian-like H1 of H1N1 as compared to the human-like H1 of rH1N1 and H1N2 SIVs. Interestingly, the Sa (13 amino acids) site is an immunodominant antigenic site in pH1N1 (Manicassamy *et al.*, 2010; Retamal *et al.*, 2014; Strengell *et al.*, 2011), and was found to be completely conserved between the first two viruses as previously reported (Castrucci *et al.*, 2014), while there were 5-6 amino acid differences between pH1N1 and the human-like H1 SIVs. This may explain why the pH1N1infection-immune pigs in our study were more optimal protected against the

avian-like H1N1 SIV. A complete cross-protection against the pH1N1 after prior infection of pigs with the avian-like H1N1 SIV has been demonstrated in a previous study (Busquets *et al.*, 2010). Likewise, prior infection or vaccination with 1918 pandemic or classical swine H1N1 virus, which differ from pH1N1 in only 1 amino acid in the Sa antigenic site, resulted in nearly complete protection from the latter virus in mice and ferrets (Kash *et al.*, 2010; Manicassamy *et al.*, 2010; O'Donnell *et al.*, 2012). Furthermore, there is strong evidence for cross-protection between pH1N1 and historical human seasonal H1N1 viruses from the 1930-40s in humans and in experimental animal models (O'Donnell *et al.*, 2012; Skountzou *et al.*, 2010; Skowronski *et al.*, 2011). Yet, these viruses have only 67-76% amino acid homology in their HA1 and as many as 5 amino acid differences in the Sa antigenic site (O'Donnell *et al.*, 2012). Some of the viruses used for prior infection and challenge in the present study also had NAs of the same lineage. This was the case for pH1N1 and European SIVs of the H1N1 subtype (H1N1 and rH1N1), as well as for the European H1N2 and H3N2 SIVs. Unlike antibodies against HA, anti-NA antibodies cannot neutralize influenza viruses, but do play a significant secondary role in protection against influenza in humans and animals (Kilbourne *et al.*, 2004; Monto & Kendal, 1973; Schulman *et al.*, 1968; Sylte *et al.*, 2007). Therefore, it is not surprising that pH1N1-immune pigs were better protected against European H1N1 than H1N2 SIVs, and that H1N2-immune pigs were better protected against H3N2 challenge than H1N1-immune pigs.

Protection between viruses of H1N1 and H3N2 subtype was clearly less robust than that between viruses of the same HA and/or NA subtype. Yet, pigs with infection-immunity to the avian-like H1N1 SIV were better protected against H3N2 than pH1N1-immune pigs. This suggests a contribution of immune responses to internal proteins, which are shared between avian-like H1N1 and human-like H3N2 SIVs but are of different origin in pH1N1, except for the M protein. In pigs that had been subsequently exposed to pH1N1 and the avian-like H1N1, protection against H3N2 was inferior to that in pigs exposed to H1N1 only, and similar to that in pigs exposed twice to pH1N1. This is most likely due to the failure of the H1N1 virus to replicate and induce specific immune responses in pH1N1-immune pigs, as indicated by the nasal virus titers and serological responses. Our data are largely in agreement with previous studies on heterosubtypic protection in pigs, ferrets, and mice (Heinen *et al.*, 2001; Hillaire *et al.*, 2011; Kreijtz *et al.*, 2007; Laurie *et al.*, 2010; Liang *et al.*, 1994;

Yetter *et al.*, 1980), none of which showed a complete protection between H1 and H3 viruses. For example, prior infection of ferrets with pH1N1 or a seasonal H1N1 virus resulted in a shorter duration of nasal excretion of seasonal H3N2 virus, i.e. 5 days instead of 7 days in unprimed influenza naïve ferrets (Laurie *et al.*, 2010; Yetter *et al.*, 1980). In mice, prior infection with H1N1 or pH1N1 did not reduce H3N2 virus titers in the lungs at 4 dpc, but titers were undetectable or reduced by 10^4-fold at 7 dpc (Hillaire *et al.*, 2011; Kreijtz *et al.*, 2007).

Although heterosubtypic protection has been frequently studied in mice, the pig model has some specific advantages for studying protection between influenza viruses. Pigs are natural hosts for a variety of genetically and antigenically diverse H1 and H3 viruses. Most SIVs have an HA derived from viruses that once circulated in humans and the pH1N1 is a shared virus between humans and swine (Van Reeth *et al.*, 2012). Nearly all SIVs are natural reassortants. As a result, the SIVs used in this study share 1 to 7 genes with each other, and this allows examination of the relative importance of each protein for protection. As pointed out before (Brookes *et al.*, 2010; Khatri *et al.*, 2010; Van Reeth *et al.*, 1998), the pathogenesis of influenza is very similar in pigs and in humans, and there are also striking similarities in their immune responses. In mice, the outcome of infection experiments depends on the mouse and virus strain used. For instance, upon infection with PR/8, DBA/2 mice showed a greater susceptibility to infection, more rapid weight loss and death, elevated cytokine production, and more severe lung histopathology than C57BL/6 mice (Srivastava *et al.*, 2009). Also, human influenza viruses generally require adaptation to be able to replicate and achieve virulence in mice, and the adapted virus may be antigenically and phenotypically very different from the initial strain (Bouvier & Lowen, 2010). Finally, mice transmit influenza viruses inefficiently and nasal virus excretion cannot be evaluated in mice (Lowen *et al.*, 2006). Pigs, in contrast, are highly susceptible to a variety of human H1 and H3 viruses in experimental settings (Landolt *et al.*, 2003; Qiu *et al.*, 2013), and unlike mice, pigs can shed high titer virus in nasal swabs for 4-6 days.

During the last few years, there have been several serologic investigations for cross-reactive antibodies against H1 and H3 SIVs in humans of different age categories (Hoschler *et al.*, 2013; Krumbholz *et al.*, 2014; Qiu *et al.*, 2015). HI antibodies against the avian-like H1N1 SIV were undetectable in approximately 90% of humans in 2009, and they showed only a minimal increase in humans with seroconversion to pH1N1

(De Marco *et al.*, 2013; Hoschler *et al.*, 2013; Perera *et al.*, 2011). This is in agreement with our finding that pH1N1 infection did not induce detectable serum HI antibodies against the avian-like H1N1. On the other hand, pH1N1infection-immune pigs showed a complete virological protection against the avian-like H1N1 SIV. This further supports the notion that infection with wild type live viruses can induce heterovariant protection in the absence of cross-reactive serum HI antibodies and argues for more *in vivo* cross-protection studies in animal models. From such studies, we conclude that humans will likely have better immune protection against European H1 than H3 viruses of swine. This is based on the presence of minimal protection against European H3N2 SIVs in pigs with infection immunity to contemporary human H3N2 viruses (Qiu *et al.*, 2013) or pH1N1, whereas infection immunity to pH1N1 seems to offer significant protection against the major H1 SIVs.

In conclusion, our study shows post-infection immunity may offer substantial cross-lineage protection against influenza viruses of the same HA and/or NA subtype. Heterosubtypic protection between viruses of different HA and NA subtypes, in contrast, appears to be weak in pigs. Because they are natural hosts for the same influenza virus subtypes as humans, pigs have some unique advantages as a model for cross-protection studies with influenza. According to our data, the global spread of pH1N1 in humans will enhance their cross-protective immunity to European H1 SIVs, making those viruses less likely to cause pandemics in the near future.

4.2.6 Acknowledgements

The authors thank Dr. Gaëlle Kuntz-Simon for providing the sw/Cotes d'Armor/0046/08 virus, as well as Lieve Sys, Melanie Bauwens, Nele Dennequin, Zeger van den Abeele and Philippe Elskens for excellent technical assistance, and Ivy Brown for proof reading the manuscript. This study was funded by the Belgian Federal Service for Public Health, Food Chain Safety and Environment (FLUCROSS, RT 09/6227) and the European Commission (FLUPIG, FP7-GA 258084).

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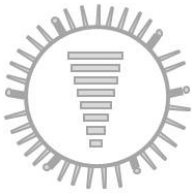

Chapter 5

Zoonotic potential of European H3N2 swine influenza viruses

Chapter 5.1

Prior infection of pigs with a recent human H3N2 influenza virus confers minimal cross-protection against a European swine H3N2 virus

Adapted from:
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Influenza Other Respir Viruses 2013, 7:1260-8.

5.1.1 Abstract

H3N2 influenza viruses circulating in humans and European pigs originate from the pandemic A/Hong Kong/68 virus. Because of slower antigenic drift in swine, the antigenic divergence between swine and human viruses has been increasing. It remains unknown to what extent this results in a reduced cross-protection between recent human and swine H3N2 influenza viruses.

We examined whether prior infection of pigs with an old [A/Victoria/3/75 (A/Vic/75)] or a more recent [A/Wisconsin/67/05 (A/Wis/05)] human H3N2 virus protected against a European swine H3N2 virus [sw/Gent/172/08 (sw/Gent/08)]. Genetic and antigenic relationships between sw/Gent/08 and a selection of human H3N2 viruses were also assessed.

After challenge with sw/Gent/08, all challenge controls had high virus titers in the entire respiratory tract at 3 days post-challenge and nasal virus excretion for 5–6 days. Prior infection with sw/Gent/08 or A/Vic/75 offered complete virological protection against challenge. Pigs previously inoculated with A/Wis/05 showed similar virus titers in the respiratory tract as challenge controls, but the mean duration of nasal shedding was 1·3 days shorter. Unlike sw/Gent/08- and A/Vic/75-inoculated pigs, A/Wis/05-inoculated pigs lacked cross-reactive neutralizing antibodies against sw/Gent/08 before challenge, but they showed a more rapid antibody response to sw/Gent/08 than challenge controls after challenge. Cross-protection and serological responses correlated with genetic and antigenic differences.

Infection immunity to a recent human H3N2 virus confers minimal cross-protection against a European swine H3N2 virus. We discuss our findings with regard to the recent zoonotic infections of humans in the United States with a swine-origin H3N2 variant virus.

5.1.2 Introduction

The antigenic and genetic characteristics of swine influenza viruses (SIVs) vary in different continents and regions of the world, but most if not all H3N2 SIVs contain hemagglutinin (HA) and neuraminidase (NA) genes from early or more recent human H3N2 viruses (Van Reeth *et al.*, 2012). In Europe, H3N2 SIVs are derived from descendants of the 1968 “Hong Kong” pandemic virus, but they have evolved further through genetic reassortment with the endemic avian-like H1N1 SIV in the mid-1980s. This has resulted in H3N2 SIVs with human-like HA and NA genes and avian-like

internal genes (Campitelli *et al.*, 1997; Castrucci *et al.*, 1993). In North America, H3N2 viruses did not become established in the pig population until 1998. These viruses are “triple” reassortants with the HA, NA, and PB1 genes of human H3N2 viruses from the mid-1990s and the remaining genes of classical swine and avian origin (Webby *et al.*, 2000). Multiple H3N2 SIV lineages have been found in Asia, and all have HA and NA genes derived from human H3N2 viruses from various time periods (Nerome *et al.*, 1995; Ngo *et al.*, 2012; Yu *et al.*, 2008). Because of slower antigenic drift in the H3 HA in swine than in humans (de Jong *et al.*, 2007; Nerome *et al.*, 1995), human-lineage H3N2 viruses in swine over time become increasingly divergent from contemporary human H3N2 viruses. As an example, European H3N2 SIVs from 1983 to 1999 showed a six times slower rate of antigenic drift than their human counterparts (de Jong *et al.*, 2007). Consequently, European H3N2 SIVs from recent years still show some degree of serological cross-reactivity with human viruses from the 1970s to 1980s, but not with human viruses isolated after 1990 (de Jong *et al.*, 1999; Kyriakis *et al.*, 2011).

Swine influenza viruses usually do not infect humans, but sporadic dead-end zoonotic infections have been reported with most SIV subtypes or lineages (Myers *et al.*, 2007; Shu *et al.*, 2012; Van Reeth, 2007). Such infections are generally mild and clinically indistinguishable from infections with human influenza viruses. Most cases of swine influenza occur in people with direct or indirect exposure to swine, frequently children, or young adults. The European H3N2 SIV has been isolated from a 1-year-old girl and a 2-year-old boy in the Netherlands in 1993 (Claas *et al.*, 1994). A closely related virus was also isolated from a 10-month-old girl in Hong Kong in 1999 (Gregory *et al.*, 2001). In North America, nine cases of human infection with triple reassortant H3N2 SIV have been reported from 2005 to 2010 (Olsen *et al.*, 2006; Robinson *et al.*, 2007; Shu *et al.*, 2012). A variant of the original triple reassortant H3N2 SIVs, which contains the matrix gene of the pandemic 2009 H1N1 virus, has been detected in 13 humans in the United States from July 2011 to April 2012 (Skowronski *et al.*, 2012), and in 306 cases in 10 US states from July to September 2012, mainly in children visiting county or state fairs (CDC, 2012b). The increasing numbers of human cases may be in part due to heightened awareness and surveillance. No H3N2 SIV has so far shown the capacity to spread efficiently between humans.

Already in 1977, Shortridge *et al*. suggested that pigs could serve as a reservoir for old, A/Hong Kong/68-like human H3N2 viruses and that such viruses might be re-introduced into humans when their immunity has waned (Shortridge *et al.*, 1977). However, it remains unknown to what extent immunity to human H3N2 viruses may offer protection against H3N2 SIVs. We have therefore examined the effect of prior infection with an old (1975) or a more recent (2005) human H3N2 influenza virus on challenge with a recent European H3N2 SIV in the pig model.

5.1.3 Materials and methods

5.1.3.1 Genetic and antigenic characterization of viruses

Sw/Gent/172/08 (sw/Gent/08) is representative of H3N2 SIVs that are enzootic in Western Europe. The human H3N2 influenza viruses A/Victoria/3/75 (A/Vic/75) and A/Wisconsin/67/05 (A/Wis/05) have been WHO vaccine reference strains in 1976–1978 and 2006–2008, respectively. All viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs for less than four passages.

The HA1, NA, matrix (M), and nucleoprotein (NP) sequences of A/Vic/75, A/Wis/05, and sw/Gent/08 were compared at the nucleotide and amino acid (aa) level using Megalign program within DNAStar 5.01 software (DNASTAR, Inc., Madison, WI, USA). In addition, the HA1 and NA of a selection of human H3N2 viruses circulating from 1973 to 2009 (Table 2) were compared with sw/Gent/08. Amino acid differences at putative antigenic sites of the HA and NA, as defined by others (Colman *et al.*, 1983; Underwood, 1982; Wiley *et al.*, 1981), were identified by alignment using Mega 5.05 software (http://www.megasoftware.net/) (Tamura *et al.*, 2011). N-linked glycosylation sites were predicted by the NetNGlyc 1.0 web server (http://www.cbs.dtu.dk/services/NetNGlyc) as described elsewhere (Das *et al.*, 2010). The HA1, NA, M, and NP of sw/Gent/08 and the HA1 of A/Vic/75 and A/Wis/05 were (re-)sequenced for this study (GenBank accession numbers KC142126–32). All other sequences used for comparison were downloaded from GenBank.

A/Vic/75, A/Wis/05, and sw/Gent/08 were characterized in hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using post-infection ferret or hyperimmune swine sera, or both.

5.1.3.2 Experimental design

Thirty-two 5-week-old pigs were obtained from an influenza virus-seronegative farm. Pigs were randomly assigned to four groups of eight pigs. Each group was housed in a separate biosafety level-2 HEPA-filtered isolation unit. Before the start of the experiment, all pigs were seronegative to any of the European endemic H1N1, H1N2, and H3N2 SIVs, as determined by HI test, immunoperoxidase monolayer assay, and a competitive anti-influenza A NP enzyme-linked immunosorbent assay (Idexx Laboratories, Hoofddorp, the Netherlands). After acclimation for 1 week, three groups were inoculated with A/Vic/75, A/Wis/05, or sw/Gent/08, respectively. One group was left uninoculated and served as the challenge control group. Six weeks later, all groups were challenged with sw/Gent/08. All inoculations were performed intranasally with 7·0 $\log_{10}$ 50% egg infective doses (EID_{50}) influenza virus in 3 ml (1.5 ml per nostril) as described elsewhere (De Vleeschauwer *et al.*, 2011). Pigs were observed daily for clinical signs from 4 days before until 7 days after each inoculation, or until euthanasia. To determine virus excretion, nasal swabs were collected daily from all pigs from 0–7 days post-primary inoculation (dpi) and from 0–7 days post-challenge (dpc), or until euthanasia. Four pigs per group were euthanized at 3 dpc and gross lung lesions were assessed as described elsewhere (Atanasova *et al.*, 2011). To determine the extent of replication of the sw/Gent/08 challenge virus in the respiratory tract, tissue samples of the upper (nasal mucosa respiratory part and olfactory part, tonsil, and trachea) and lower (apical, cardiac, and diaphragmatic lobes of the left and right lung) respiratory tract were collected and titrated separately. Blood samples for serological examinations were collected at the start of the experiment, 14 and 42 dpi, that is, at the time of challenge with sw/Gent/08. The remaining pigs were also bled at 5, 7, 10, and 14 dpc.

5.1.3.3 Virus titration

Cotton swabs were weighed before and after collection to determine virus titers per 100 mg nasal secretions. Swabs from both nostrils were suspended in 1 ml of phosphate-buffered saline supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin and mixed vigorously at 4°C for 1 hour. Tissue samples were weighed and ground in PBS containing 10 IU/ml penicillin and 10 µg/ml streptomycin to obtain 20% (w/v) tissue homogenates. Nasal swab samples and tissue homogenates were clarified by centrifugation (16 000 *g* for 3 minutes) and stored at −70°C until titration. All samples were titrated on Madin–Darby canine

kidney (MDCK) cells in serum-free medium with trypsin. Briefly, confluent monolayers of cells were inoculated with 10-fold serial dilutions of samples. Cells were washed 2 hours after inoculation and subsequently observed for development of cytopathic effect over 7 days. Virus titers were expressed as $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$) per 100 mg (nasal swabs) or per gram (tissues).

5.1.3.4 Serological assays

Serum antibody responses were examined by HI, virus neutralization (VN), and NI tests. All sera collected at 0, 14 dpi, and 0 and 14 dpc were examined against all three viruses in HI and VN tests, while only the sera collected at 0 and 14 dpc were tested in NI tests. Additional VN tests against the challenge virus sw/Gent/08 were performed on the sera collected at 5, 7, and 10 dpc. All sera were heat inactivated (56°C, 30 minutes) before use. The HI test was performed according to standard procedures with 0·5% turkey erythrocytes and 4 hemagglutinating units of virus (Leuwerke *et al.*, 2008). The VN test was performed in MDCK cells in microplates with 100 $TCID_{50}$ of virus per well as previously described (Van Reeth *et al.*, 2003). The NI test was based on the colorimetric analysis of sialic acid release from fetuin substrate and conducted in 96-well PCR plates as described elsewhere (Sandbulte *et al.*, 2009). Starting dilutions were 1:2 in the VN test and 1:10 in the HI and NI tests.

5.1.3.5 Statistics

Nasal virus shedding in each group was quantified by calculation of the area under the curve (AUC), which is obtained by plotting viral titers versus each time point of sample collection. Samples that tested negative for virus were given a numeric value of 1·6 $\log_{10}$ $TCID_{50}$ per 100 mg or gram. Samples that tested negative in the serological assays were assigned a value corresponding to half of the minimum detectable titer. Mann–Whitney tests were used to compare virus titers and antibody levels between any two experimental groups. $P < 0.05$ was considered statistically significant. Graphpad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for all analyses.

5.1.4 Results

5.1.4.1 Genetic and antigenic relationships between human and European swine H3N2 viruses

Percentages of nucleotide and aa identity between the HA1, NA, M, and NP of A/Vic/75, A/Wis/05, and sw/Gent/08 are summarized in Table 1. The HA1 and NA of sw/Gent/08 were more similar to A/Vic/75 than to A/Wis/05 at both levels. The M and NP genes of both human viruses were equally similar to those of the swine virus.

Table 1. Percent identity of the nucleotide and amino acid sequences of the hemagglutinin (HA1), neuraminidase (NA), matrix (M), and nucleoprotein (NP) genes of sw/Gent/08 with those of the human H3N2 viruses A/Vic/75 and A/Wis/05

	% identity compared to sw/Gent/08							
	HA1		NA		M		NP	
	N	aa	N	aa	N	aa	N	aa
A/Vic/75	88	87	89	91	87	91	84	91
A/Wis/05	80	80	85	87	86	90	82	91

N, nucleotide; aa, amino acid.

Amino acid differences at presumed antigenic sites of the HA are shown in Figure 1. The HA1 segment of A/Vic/75 contained 42 aa differences compared with sw/Gent/08, with 9 of these occurring in each of the 5 recognized antigenic sites: 4 in antigenic site A, 1 in B, 2 in C, 1 in D, and 1 in E. The HA1 of A/Wis/05 contained as much as 64 aa differences compared with sw/Gent/08. Fourteen differences were located in antigenic sites: 2 in antigenic site A, 7 in B, 3 in C, 1 in D, and 1 in E. Three additional N-glycosylation sites in the globular head of HA1, at positions 133, 144, and 246, were found in A/Wis/05 but absent in A/Vic/75 and sw/Gent/08. Positions 133 and 144 were located in antigenic site A and position 133 was in the receptor binding site. The NA gene of A/Vic/75 was also more closely related to sw/Gent/08 (44 aa differences, with 10 in putative antigenic sites) than that of A/Wis/05 (62 aa differences, 15 in antigenic sites). Compared with sw/Gent/08, one additional N-glycosylation site was predicted for A/Vic/75 (position 234) and two for A/Wis/05 (positions 93 and 234).

Table 2 compares the HA1 and NA protein sequences and presumed antigenic sites of sw/Gent/08 with those of additional human H3N2 viruses collected over time. The HA1 of sw/Gent/08 was most similar to that of A/Vic/75. Starting in 1989, all human

strains showed less than 85% aa homology with the swine virus, and 14–17 aa differences in antigenic sites of the HA. N-glycosylation site 246 and 133 were consistently present in the globular head of the HA1 of all human H3N2 viruses examined since 1987 and 1997, respectively. The NA of sw/Gent/08 was most similar to that of A/Port Chalmers/1/73. Human viruses from 1989 or later showed <89% aa homology with the swine virus and 13–15 aa differences in antigenic sites of the NA.

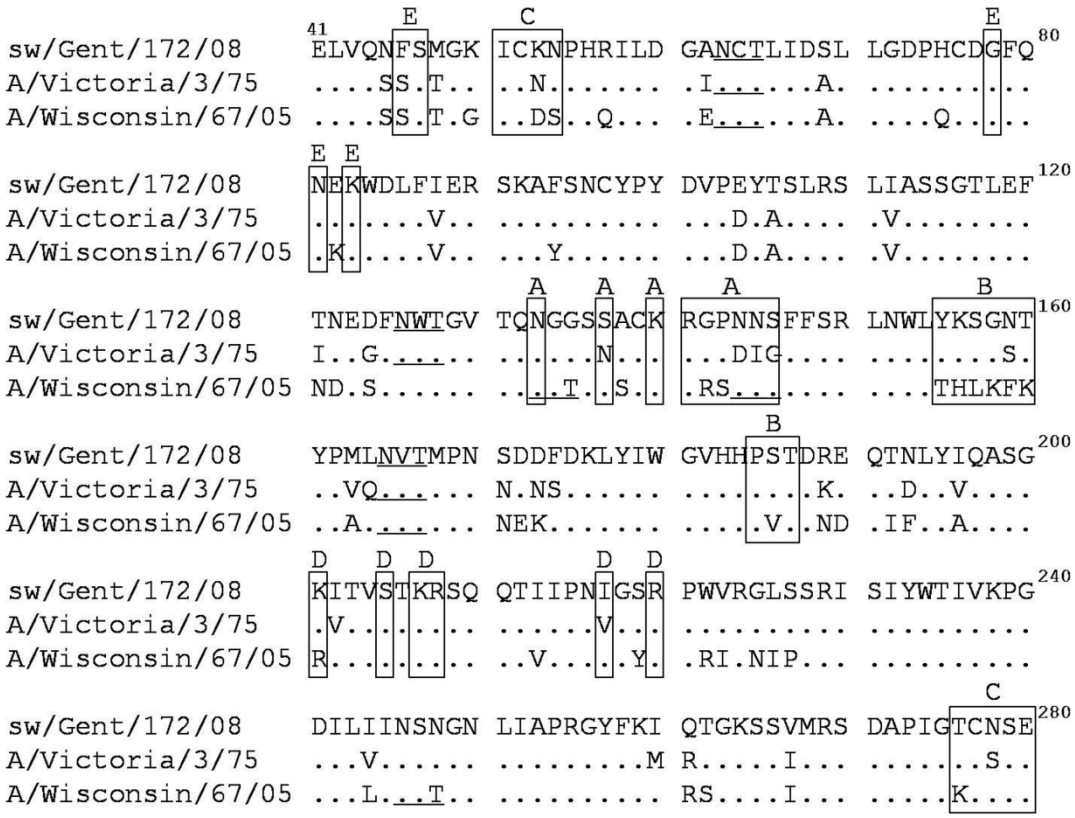

Figure 1. Alignment of deduced amino acid sequences in the HA1 of sw/Gent/08, A/Vic/75, and A/Wis/05. Residues in the open boxes represent previously identified antigenic sites (A, B, C, D, and E) of H3. Underlined residues represent potential N-glycosylation sites in the globular head of the HA1. Only amino acids different from those in the sw/Gent/08 sequence are shown, conserved residues are shown as dots.

Table 2. Comparison of hemagglutinin (HA1) and neuraminidase (NA) protein sequences of epidemic human H3N2 influenza viruses from 1973 to 2009 and their antigenic sites with those of sw/Gent/08

	HA1			NA		
Virus strain	Genbank accession no.	% aa identity	No. of aa differences in antigenic sites	Genbank accession no.	% aa identity	No. of aa differences in antigenic sites
A/Port Chalmers/1/73	ABE12532	86.9	11	ABE12548	91.6	6
A/Victoria/3/75	AFY08275	87.2	9	AAB03361	90.5	10
A/Texas/1/77	ABQ58940	86.9	11	AFM68968	90.7	9
A/Bangkok/01/79	ABF21268	85.4	13	ABF21324	90.3	9
A/Philippines/2/82	ADJ41805	85.4	11	ADJ41808	89.7	10
A/Leningrad/360/86	AAB69845	85.1	13	AFN11845	89.7	11
A/Sichuan/02/87	D10161	85.1	15	-	-	-
A/Beijing/353/89	AAB58297	83.9	16	AAB06969	88.6	14
A/Shangdong/9/93	ACL12129	82.4	16	ACL12132	88.6	13
A/Wuhan/359/95	AFR42694	83.0	16	AAB06998	87.7	13
A/Sydney/5/97	ACO95259	81.8	17	ACO95262	88.1	13
A/Moscow/10/99	ABE73115	82.7	16	ABE73101	86.4	15
A/California/7/04	ABO37490	81.2	14	ABV24038	86.9	15
A/Wisconsin/67/05	AFY08274	80.2	14	ABW80983	86.6	15
A/Brisbane/10/07	ABW23353	81.2	15	ACO95273	86.2	14
A/Perth/16/09	ACS71642	80.9	16	ADW80519	86.0	14

-: not available in Genbank.

Table 3 shows the antigenic relationship in the HA and NA of the three viruses used in infection-challenge studies. Low to moderate cross-reactivity between A/Vic/75 and sw/Gent/08 was observed in both HI and NI tests, whereas antisera against the two viruses lacked cross-reactivity with A/Wis/05 in both tests. For an unknown reason, ferret serum against A/Wis/05 showed similar NI antibody titers to all three viruses, while the swine serum showed minimal reaction with sw/Gent/08 or A/Vic/75.

Table 3. Cross-reactivity between the human H3N2 viruses A/Vic/75 and A/Wis/05 and the swine H3N2 virus sw/Gent/08 in hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays

		Antibody titers with serum to…			
		A/Vic/75 (F)*	A/Wis/05 (F)	A/Wis/05 (S)*	sw/Gent/08 (S)
A/Vic/75	HI	160	< 10	< 10	40
	NI	2560	40	< 10	320
A/Wis/05	HI	< 10	2560	2560	< 10
	NI	< 10	80	640	< 10
sw/Gent/08	HI	40	< 10	<10	1280
	NI	320	40	20	5120

* (F): post-infection ferret serum, (S): hyperimmune swine serum.

5.1.4.2 Virus excretion and serological response after primary inoculation

As expected after intranasal inoculation of pigs with influenza virus,[1] clinical symptoms were absent in most pigs except for two sw/Gent/08-inoculated pigs with dyspnea at 3 dpi. All pigs excreted virus in nasal swabs. Virus shedding was detected for 5–6 consecutive dpi with sw/Gent/08 or A/Vic/75 and for 4–5 dpi with A/Wis/05. Mean virus titers in nasal swabs are shown in Figure 2. The average AUC value was highest for sw/Gent/08 (21.8), followed by A/Vic/75 (16.6) and A/Wis/05 (12.1).

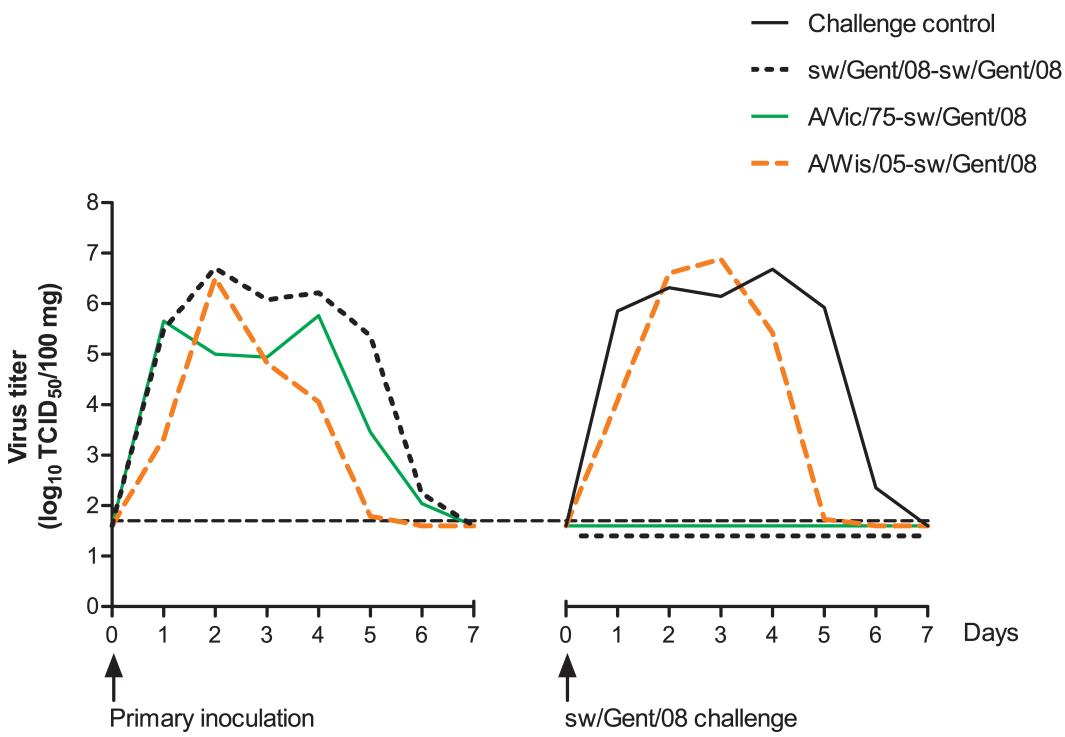

Figure 2. Nasal virus excretion after primary inoculation with human or swine H3N2 influenza virus and after challenge with sw/Gent/08. Mean virus titers in nasal swabs of each group are given. The horizontal broken line represents the detection limit (<1.7 $\log_{10}$ $TCID^{50}$/100 mg).

All pigs were seronegative against the three viruses prior to the start of the experiment. Antibody titers at 2 and 6 weeks after the primary inoculation are shown in Table 4. All challenge controls remained seronegative until the time of challenge, whereas the other pigs developed HI, VN, and NI antibodies to the influenza virus used for inoculation. These homologous antibody titers were significantly higher in sw/Gent/08-inoculated pigs than in the pigs inoculated with human H3N2 viruses ($P < 0.05$), except for the HI titers at 14 dpi ($P > 0.05$). Inoculation with A/Vic/75 induced cross-reactive VN antibodies against sw/Gent/08 in all pigs. Cross-reactive HI antibodies were detected in only 3 of 8 pigs, at 14 dpi only, and cross-reactive NI antibodies in 6 pigs. In A/Wis/05-inoculated pigs, serological cross-reaction with sw/Gent/08 was negligible in the VN test and undetectable in the HI or NI test.

Table 4. Antibody response before and after challenge with sw/Gent/08 in challenge controls and pigs first infected with human or swine H3N2 influenza virus

		Geometric mean antibody titers of positive pigs (no. of positive pigs/total no.)								
		14 days post first inoculation			Time of challenge			14 days post challenge		
Group		A/Vic/75	A/Wis/05	sw/Gent/08	A/Vic/75	A/Wis/05	sw/Gent/08	A/Vic/75	A/Wis/05	sw/Gent/08
Challenge control	HI	<10	<10	<10	<10	<10	<10	12(4/4)	<10	160(4/4)
	VN	<2	<2	<2	<2	<2	<2	20(4/4)	<2	347(4/4)
	NI	n.d.	n.d.	n.d.	<10	<10	<10	34(4/4)	<10	381(4/4)
sw/Gent/08-sw/Gent/08	HI	13(5/8)	<10	104 (8/8)	10(4/8)	<10	52(8/8)	10(1/4)	<`0	57(4/4)
	VN	9(8/8)	2(1/8)	268(8/8)	5(8/8)	2(4/8)	206(8/8)	12(4/4)	3(3/4)	292(4/4)
	NI	n.d.	n.d.	n.d.	17(5/8)	<10	320(8/8)	25(3/4)	<10	269(4/4)
A/Vic/75-sw/Gent/08	HI	52(8/8)	<10	13(3/8)	13(8/8)	<10	<10	67(4/4)	<10	80(4/4)
	VN	83(8/8)	2(3/8)	15(8/8)	58(8/8)	3(3/8)	14(8/8)	491(4/4)	5(2/4)	457(4/4)
	NI	n.d.	n.d.	n.d.	20(8/8)	<10	13(6/8)	381(4/4)	<10	269(4/4)
A/Wis/05-sw/Gent/08	HI	<10	80(8/8)	<10	<10	24(8/8)	<10	14(4/4)	453(4/4)	226(4/4)
	VN	2(3/8)	41(8/8)	4(1/8)	2(1/8)	27(8/8)	3(2/8)	17(4/4)	431(4/4)	264(4/4)
	NI	n.d.	n.d.	n.d.	<10	10(8/8)	<10	135(4/4)	381(4/4)	1076(4/4)

HI, hemagglutination inhibition; VN, virus neutralization; NI, neuraminidase inhibition; n.d., not determined.

5.1.4.2 Virological protection after challenge with sw/Gent/08

No clinical signs were observed in any pigs after challenge infection. Mean sw/Gent/08 titers in nasal swabs are shown in Figure 2. Virus shedding was detectable in all challenge controls during 5–6 consecutive days or until euthanasia. All pigs of the sw/Gent/08-sw/Gent/08 and A/Vic/75-sw/Gent/08 groups tested negative for virus excretion. Pre-infection with A/Wis/05 failed to prevent challenge infection or to reduce peak virus titers in nasal swabs. However, the mean duration of nasal shedding was 1·3 days shorter than in the challenge control group.

Sw/Gent/08 virus titers in the respiratory tract of the pigs euthanized at 3 dpc are shown in Table 5. Sw/Gent/08 was isolated from the nasal mucosa, tonsil, trachea, left lung, and right lung of all challenge control pigs. In contrast, all pigs of the sw/Gent/08-sw/Gent/08 and A/Vic/75-sw/Gent/08 groups were completely virus negative. In the A/Wis/05-sw/Gent/08 group, virus isolation rates and virus titers were similar as in the challenge control group ($P > 0.05$). Lung lesions characterized by dark-red consolidated areas, involving only 1–2% of the lung surface, were present in two challenge control pigs and two A/Wis/05-sw/Gent/08 pigs, but absent in both other groups.

5.1.4.3 Serological profile after challenge with sw/Gent/08

All pigs of the challenge control group had developed HI, VN, and NI antibodies to sw/Gent/08 at 14 dpc (Table 4), whereas anti-sw/Gent/08 antibody titers remained at pre-challenge levels in the sw/Gent/08-sw/Gent/08 group ($P > 0.05$). Pigs of the A/Vic/75-sw/Gent/08 and A/Wis/05-sw/Gent/08 groups developed antibodies to sw/Gent/08 or showed a considerable increase in pre-existing antibody titers. As shown in Figure 3, VN antibodies to the challenge virus developed more rapidly in the A/Wis/05-sw/Gent/08 group than in the challenge control group, but all 4 groups had similar antibody titers at 10 dpc. Homologous antibody titers to the human H3N2 viruses also increased after challenge with sw/Gent/08. Many pigs showed low cross-reactive antibody titers to A/Vic/75 without being exposed to it, but cross-reaction with A/Wis/05 was rare.

Table 5. Virus titers in the respiratory tract 3 days after challenge with sw/Gent/08 in challenge controls and pigs first infected with human or swine H3N2 influenza virus

	Mean virus titers (log_{10} $TCID_{50}$/g) ± SEM*					
Group	Nasal mucosa respiratory part	Nasal mucosa olfactory part	Tonsil	Trachea	Left lung	Right lung
Challenge control	6.4 ± 0.1	5.4 ± 0.5	4.1 ± 0.7	7.1 ± 0.1	6.2 ± 0.1	6.7 ± 0.3
sw/Gent/08-sw/Gent/08	<†	<	<	<	<	<
A/Vic/75-sw/Gent/08	<	<	<	<	<	<
A/Wis/05-sw/Gent/08	7.6 ± 0.4	4.6 ± 1.1	4.6 ± 0.7	7.3 ± 0.2	6.2 ± 0.3	6.1 ± 0.3

* Standard error of the mean; † Virus titers below the detection limit (<1.7 log_{10} $TCID_{50}$/g)

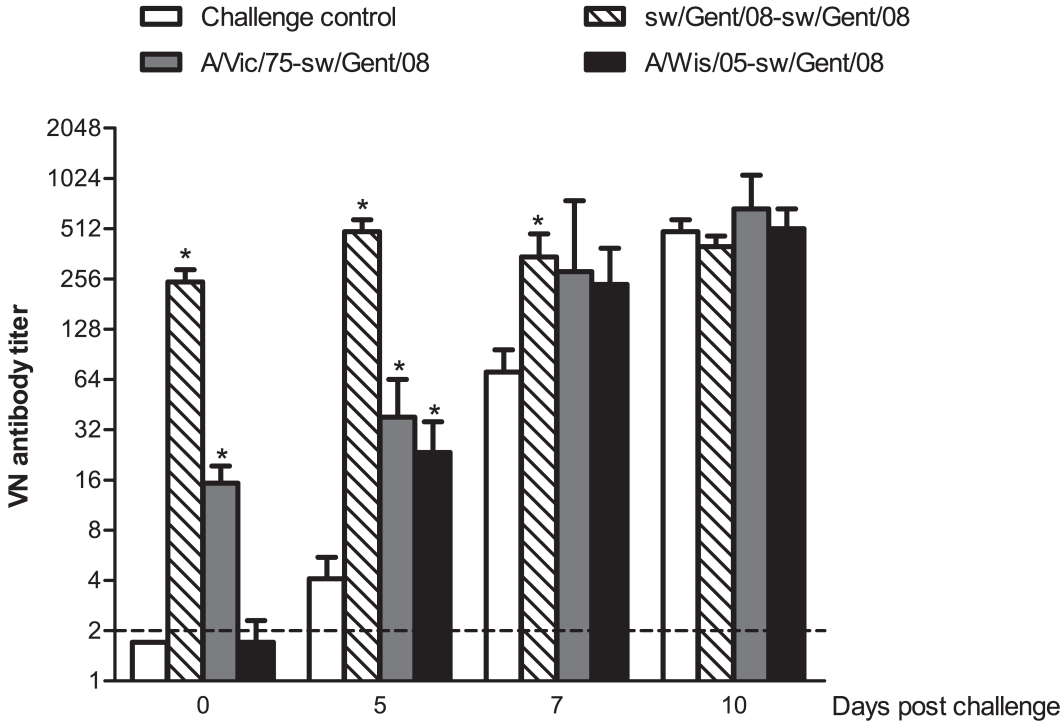

Figure 3. Evolution of virus-neutralizing (VN) antibody titers against the challenge virus sw/Gent/08 during the first 10 days post challenge. Bars represent group geometric mean VN antibody titers with standard error of the mean (SEM); the asterisk denotes a statistically significant difference ($P < 0.05$) with the challenge control group. The horizontal broken line represents the detection limit of the assay (<2).

5.1.5 Discussion

The increasing antigenic divergence between H3N2 viruses from swine and humans may have important implications for zoonotic transmission of H3N2 SIVs. This is the

first study to evaluate cross-protection between human and swine H3N2 viruses in pigs. It shows a complete virological protection against infection with a contemporary European H3N2 SIV in pigs infected with A/Vic/75 6 weeks earlier, but only a minimal protection in pigs pre-infected with the more recent human H3N2 virus A/Wis/05. We did not identify the immune mechanisms underlying the observed cross-protection, but several findings point toward a role for cross-reactive antibodies against the HA and/or NA of the swine virus. Indeed, the difference in protective immune response induced by the two human viruses was in line with the genetic and antigenic differences in their HA and NA. Antigenic sites A and B are located on the tip of the HA molecule, and they are supposed to be the primary targets for neutralizing antibodies (Underwood, 1982; Wiley *et al.*, 1981). A/Wis/05 differed from sw/Gent/08 in as much as seven of the total nine amino acids of site B, and it had two additional N-glycosylations in site A, which make it more distinct from sw/Gent/08 than A/Vic/75. In addition, low-titered cross-reactive VN antibodies against sw/Gent/08 were found in all pigs previously infected with A/Vic/75, but they were rare after infection with A/Wis/05. Post-challenge, however, such antibodies developed more rapidly in A/Wis/05 pre-infected pigs than in challenge controls, and this coincided with the enhanced clearance of the challenge virus. The HI test, as well as the NI test, is less sensitive than the VN test (De Vleeschauwer *et al.*, 2011). This can explain the lack of cross-reactive HI and NI antibodies in most A/Vic/75-inoculated pigs. Given the low cross-reactive serum antibody titers in A/Vic/75-inoculated pigs, it is likely that local antibodies in the respiratory tract and cell-mediated immunity, which are more cross-reactive (Tamura & Kurata, 2004), also contribute to the cross-protection.

Previous studies by Kyriakis *et al*. (Kyriakis *et al.*, 2011) have shown that H3N2 SIVs from 2007 to 2008 cross-react in the HI test with hyperimmune sera against the human viruses A/Vic/75 and A/Philippines/2/82, but not with sera against A/Sydney/5/97 or A/Wis/05. The present comparison of sw/Gent/08 with human H3N2 viruses showed increased genetic differences since 1987–1989, as reflected by an increased number of aa differences in the HA (≥14) and NA (≥13), and additional N-glycosylations in the HA1 globular head. This suggests that especially people born after the mid-1980s are at a higher risk for infection with European H3N2 SIVs. Additional *in vivo* cross-protection studies with more, antigenically distinct human viruses would be required for a better understanding of the nature of the human viruses that could offer cross-protection against swine viruses. Still, genetic

and antigenic analyses should be interpreted with caution. Our cross-NI test results with A/Wis/05 serum from ferrets versus swine, for example, illustrate that sera from different animal species may yield discrepant results. As for the definition of antigenic sites, this is based on studies of the oldest human viruses and their reaction with mouse monoclonal antibodies. It is questionable whether the antigenic sites of such historical viruses will overlap exactly with those of recent human or swine H3N2 viruses. Furthermore, different animal species will likely mount antibodies recognizing different epitopes. Finally, some amino acids will be more important for antigenic drift than others. According to recent studies, only 7 aa positions in the HA were largely responsible for the antigenic evolution of human H3N2 viruses over a period of 35 years (Koel *et al.*, 2013). It is of great interest to identify the immunodominant amino acids in the HA of SIVs and those that are cross-reactive with human H3N2 viruses.

Virus titers in nasal swabs and antibody titers in serum were lower in pigs inoculated with human H3N2 virus than in pigs inoculated with sw/Gent/08. A/Wis/05 also seemed to replicate less efficiently in pigs than A/Vic/75, which is more closely related to swine-adapted H3N2 viruses. Similar findings were made in a comparative pig infection study with a triple reassortant H3N2 SIV from North America and a non-reassortant wholly human H3N2 virus (Landolt *et al.*, 2003). Like the authors of that study, we suspect that the lower antibody responses to human influenza viruses in pigs are in part due to their lower replication efficiencies. Virus titers in the ferret model also differ for different H3N2 virus strains (Svitek *et al.*, 2008; van den Brand *et al.*, 2012). Comparative studies of the pathogenesis of human and swine H3N2 viruses in pigs and ferrets would help to select the best animal models and experimental conditions for cross-protection studies with such viruses. On the other hand, a separate experiment in our laboratory has demonstrated complete protection against A/Wis/05 in pigs that had been previously infected with the homologous virus under the same conditions as in the present study, and with similar antibody titers. Thus, human H3N2 viruses can elicit an efficient protective immune response in pigs.

The recent infections of over 300 humans in the United States, mainly children in a fair setting, with a H3N2 variant virus (H3N2v) of swine origin have raised concerns about the extent of pre-existing immunity against this virus in the human population. Serological studies have shown cross-reactive HI antibodies in ≥50% of late adolescents and young adults, whereas such antibodies were generally lacking in

children under 10 (CDC, 2012a; Skowronski *et al.*, 2012; Waalen *et al.*, 2012). Overall, these serological findings and the present study support the idea that various H3N2 SIV lineages could cause epi- or pandemics in the younger population, should they acquire the capacity to spread efficiently between people. The H3 of H3N2v is phylogenetically most closely related to human H3N2 viruses from the 1995 to 1997 era and slightly more related to human H3N2 viruses from the 21st century than European H3N2 SIVs (Lina *et al.*, 2011). We therefore assume that the immune status of young people may be even lower for the European H3N2 SIV than for H3N2v. Both serological investigation of humans and experimental cross-protection studies, as well as studies of the host barriers, are needed to further explore the susceptibility of humans to various H3N2 SIVs. Experimental cross-protection studies are performed under artificial conditions, but they will assess all arms of the immune response and true protection. Many serological studies only consider subjects with HI titers ≥40 as protected, and they may therefore underestimate protection against SIVs in the human population.

5.1.6 Acknowledgements

We thank Dr. John McCauley for providing the human H3N2 influenza viruses, Prof. Dr. Guus Rimmelzwaan for providing the ferret sera, and Lieve Sys, Melanie Bauwens, Philippe Elskens and Zeger van den Abeele for excellent technical assistance. This study was supported by the Belgian Federal Public Service Health, Food Chain Safety, and Environment (RT 09/6227, FLUCROSS) and the European Commission (FP7-GA 258084, FLUPIG).

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Chapter 5.2

Lower seroreactivity to European than to North American H3N2 swine influenza viruses in humans, Luxembourg, 2010

Adapted from:
Yu Qiu, Claude P Muller, Kristien Van Reeth
Euro Surveill 2015, 20:25-33.

5.2.1 Abstract:

Seroreactivity to H3N2 swine influenza viruses (SIVs) was evaluated in serum samples collected from 843 people aged 0 to 100 years in 2010 in Luxembourg. Sera were analyzed by hemagglutination inhibition (HI) and virus neutralization (VN) assays targeting a European H3N2 SIV, a North American H3N2 variant of swine origin (H3N2v) and human seasonal H3N2 viruses isolated in 1975, 1995 and 2005. HI antibodies (titer ≥ 10) against European H3N2 SIV were almost exclusively detected in those born before 1990, of whom 70% were seropositive. HI antibodies against H3N2v were predominantly found in those born before 2000, with 86% seropositive. Titres against the North American H3N2v were higher than against the European H3N2 SIV. VN patterns were similar, but with higher rates and titers. We also demonstrated lower seroreactivity to European H3N2 SIV than to North American H3N2v virus. Finally, we found a strong correlation between HI titers against the European H3N2 SIV and H3N2v and their respective human ancestors, A/Victoria/3/75 and A/Nanchang/933/95. This finding and the minimal contacts between humans and pigs in Luxembourg suggest that anti-SIV antibodies in human serum samples reflect serological cross-reactivity with historical human H3N2 viruses. Our findings help assess the pandemic risk of H3N2 SIV.

5.2.2 Introduction

Three swine influenza virus (SIV) subtypes, H1N1, H1N2 and H3N2, are enzootic throughout the world in regions with a high density of pigs. The hemagglutinin (HA) and neuraminidase (NA) genes of most, if not all, H3N2 SIVs have been derived from human seasonal influenza A(H3N2) viruses. In Europe, H3N2 SIVs are derived from descendants of the A/Hong Kong/1/68 pandemic influenza A(H3N2) virus, but they have evolved further through genetic reassortment with the endemic avian-like H1N1 SIVs present in western Europe since the late 1970s. This has resulted in H3N2 SIVs with human-like HA and NA genes and avian-like internal genes (Campitelli *et al.*, 1997; Castrucci *et al.*, 1993). In North America, H3N2 viruses have become established in swine since 1998. They are known as ‘triple-reassortant’ viruses because their HA, NA and polymerase B1 genes stem from human seasonal H3N2 viruses and the remaining internal genes from avian influenza virus and classical H1N1 SIV (Webby *et al.*, 2000). Since 2009, novel reassortant H3N2 viruses with

variable numbers of internal genes derived from the 2009 pandemic influenza pH1N1 virus have been reported frequently and this has further complicated the epidemiology of swine influenza in the United States (US) (Kitikoon *et al.*, 2013). From 2009 to 2012, these novel influenza pH1N1 reassortants accounted for 54% of H3N2 SIVs isolated (Kitikoon *et al.*, 2013). Reassortant viruses with seven genes from the triple-reassortant H3N2 SIVs and only the matrix (M) gene from the pH1N1 virus have become the dominant genotype. These viruses, called H3N2 variant or H3N2v when isolated from humans, have caused many zoonotic infections since 2011 (Lindstrom *et al.*, 2012).

Antigenic drift in the HA is generally slower in SIVs than in human influenza viruses, and pigs can therefore serve as reservoirs of older human HAs (de Jong *et al.*, 2007; Kyriakis *et al.*, 2011; Shortridge *et al.*, 1977). Swine-adapted viruses with an HA of human origin could initiate a pandemic once immunity within the human population has waned sufficiently to allow widespread infection, provided that the viruses also have the ability to spread efficiently from person to person. This was observed for the pH1N1 virus that contains the classical swine H1. Evolutionarily, the 1918 H1N1 pandemic influenza virus was the common ancestor of human seasonal and classical swine H1N1 influenza viruses; it has undergone significant antigenic drift in humans but remained largely in antigenic stasis in swine (Kash *et al.*, 2010; Skountzou *et al.*, 2010). Consequently, only people born before the 1940s had been previously exposed to human seasonal H1N1 viruses with an H1 related to that of pH1N1, and a pandemic was possible because younger people lacked cross-reactive anti-H1 antibodies (CDC, 2009; Hancock *et al.*, 2009; Ikonen *et al.*, 2010a; Skowronski *et al.*, 2011). A similar situation could occur with human-adapted H3N2 SIVs in the future if they carry the HA of seasonal H3N2 viruses that have not circulated in decades.

Before 2011, only sporadic dead-end zoonotic infections with H3N2 SIVs had been reported, in humans in close contact with pigs. Recently, the H3N2v virus has caused 343 human infections in the US from August 2011 through October 2014 (CDC, 2015). These infections occurred primarily in young children visiting agricultural fairs, and the H3N2v virus did not spread widely through the human population (Jhung *et al.*, 2013). These zoonotic infections prompted serological investigations for cross-reactive antibodies against H3N2v in people of various ages in the US, Canada, Norway and England. These studies found that more than half of the adolescents and young adults tested had hemagglutination inhibition (HI) antibody titers ≥ 40,

which is considered as seroprotective (Potter & Oxford, 1979). In contrast, younger children and older adults typically exhibited lower or negative antibody titers (CDC, 2012; Hoschler *et al.*, 2013; Skowronski *et al.*, 2012a; Skowronski *et al.*, 2012b; Waalen *et al.*, 2012).

Antibodies against the antigenically distinct European H3N2 SIV have been reported in ca 50% of humans in studies in Italy and Germany between 2008 and 2010 (De Marco *et al.*, 2013; Krumbholz *et al.*, 2010). However, these studies sought to compare antibody prevalences in swine workers and non-swine workers with a mean age of 45 years, rather than using an age-stratified design to assess seropositivity in the general population. In this study, we primarily sought to compare the seroreactivity to a European H3N2 SIV with that to a North American H3N2v virus in people in various age groups in Luxembourg who were very unlikely to have been exposed to pigs. Importantly, we also examined the association between antibody titers against swine-origin and those against human seasonal H3N2 influenza viruses.

5.2.3 Material and methods

5.2.3.1 Serum samples

A total of 843 anonymised human serum samples were randomly selected from the Serum Bank of the Laboratoire National de Santé, Luxembourg. The sera were collected from patients admitted to hospital for various reasons in April or May 2010. The sera were from people born between 1910 and 2010 and were divided into 10 groups by birth decade. As an example, 1910s refers to people born between 1910 and 1919. Ca 10 sera per year of birth and 100 sera per birth decade were tested. Only the youngest (n = 40 samples) and oldest age group (n = 9 samples) had fewer samples. The sex ratio in each age group was ca 50:50, except for the participants born in the 1910s (two male vs seven females). No further personal data were collected due to ethical constraints.

5.2.3.2 H3N2 influenza viruses

We measured serum antibody titers against human seasonal H3N2 influenza viruses A/Victoria/3/75, A/Nanchang/933/95 and A/Wisconsin/67/05. These viruses were circulating worldwide during 1976–78, 1996–98 and 2006–08, respectively, and were recommended as the influenza vaccine strains by the World Health Organization

during their time of circulation. We also measured antibody titers against sw/Gent/172/08, a virus representative of H3N2 SIVs that are currently circulating in western Europe, and against A/Indiana/08/11, which represents swine-origin H3N2v viruses isolated from humans in the US since 2011.

The HA1 amino acid sequences of these five viruses, a selection of human seasonal influenza A(H3N2) viruses (1968–2012), and European and North American H3N2 SIVs (1984–2012) were downloaded from GenBank. The sequences were compared using the MegAlign programme with DNASTAR 5.01 software (DNASTAR, Inc., Madison, WI, US). A neighbour-joining phylogenetic tree was constructed to compare amino acid sequences with MEGA 5.05 software (http://www.megasoftware.net/). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are expressed as the number of amino acid substitutions per site.

Antigenic characterisation of the five viruses used for serology was performed by HI and virus neutralization (VN) assays with hyperimmune swine serum (for sw/Gent/172/08) or post-infection ferret sera (for all other viruses). We used ferret serum against A/Wuhan/359/95 instead of A/Nanchang/933/95 because a ferret serum against the latter virus was not available. The two viruses have only two amino acid differences in the HA1, outside the antigenic region. Viruses used in HI assays were propagated in 10 day-old embryonated chicken eggs (≤ 4 passages); viruses used in VN assays underwent an additional passage in Madin-Darby canine kidney (MDCK) cells.

5.2.3.3 Serological assays

All sera were examined in HI assays against the three human seasonal H3N2 viruses and the two swine-origin H3N2 viruses. Since the VN assay is more sensitive than the HI assay and highly relevant for protection, sera from people born after 1940 were also tested in VN assays against the swine-origin viruses. HI and VN assays were performed following standard procedures (Leuwerke *et al.*, 2008; Van Reeth *et al.*, 2003). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete inhibition of HA of 4 hemagglutinating units of virus (HI assay), or 50% neutralization of 10^2 50% tissue culture infectious doses ($TCID_{50}$) of virus in MDCK cells (VN assay). The starting serum dilution was 1:10 for both

assays. Sera with titers ≥ 10 were considered as seropositive. HI titers ≥ 40 were considered as seroprotective.

5.2.3.4 Statistical analysis

Geometric mean titers (GMTs) of antibody with 95% confidence intervals (CI) were calculated for each age group against each of the five influenza viruses. A numeric value of 5 was assigned to samples with antibody titers < 10. Antibody titers between age groups were compared using the nonparametric Wilcoxon signed-rank test, and differences in the proportion of sera with HI titers ≥ 40 were analyzed using Fisher's exact test. A p value < 0.05 was considered statistically significant. Pearson correlation tests were used to compare HI antibody titers against human and swine-origin viruses. All analyses were performed with Graphpad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, US).

5.2.4 Results

5.2.4.1 Relationships between human seasonal and swine-origin influenza A(H3N2) viruses

Phylogenetic relationships between the HA1 amino acid sequences of human seasonal H3N2 viruses and H3N2 SIVs from Europe and North America are shown in Figure 1. European and North American H3N2 SIVs formed separate clusters, and branched off from human viruses at different apparent time points. The HA1 of the human A/Victoria/3/75 virus was most closely related to the European H3N2 SIVs (87.0% identity to sw/Gent/172/08). The HA1 of the human A/Nanchang/933/95 virus was most closely related to North American H3N2 SIVs (89.7% identity to A/Indiana/08/11).

Antigenic relationships between human seasonal and swine-origin H3N2 viruses are shown in Table 1. All viruses reacted with their homologous antisera at HI and VN titers ≥ 160. There was minimal cross-reactivity between the H3N2v virus A/Indiana/08/11 and the European H3N2 SIV sw/Gent/172/08 using hyperimmune swine serum against sw/Gent/172/08, and ferret serum against A/Indiana/08/11 failed to cross-react with sw/Gent/172/08. Sw/Gent/172/08 showed cross-reaction with antiserum against the human A/Victoria/3/75 virus in both HI and VN assays (titer = 80). A/Indiana/08/11, on the other hand, reacted with antiserum against the human A/Wuhan/359/95 virus, which is similar to A/Nanchang/933/95, in the VN

assay (titer = 240), but not in the HI assay. Both swine-origin viruses had negligible cross-reactivity with A/Wisconsin/67/05.

Table 1. Cross-reactivity between human seasonal A(H3N2) viruses (A/Victoria/3/75, A/Nanchang/933/95 and A/Wisconsin/67/05) and swine-origin A(H3N2) viruses (sw/Gent/172/08 and A/Indiana/08/11) in hemagglutination inhibition and virus neutralization assays, Luxembourg, 2010 (n = 843)

Virus strain	Antibody titers with serum to ...									
	A/Victoria/3/75		A/Wuhan/359/95[b]		A/Wisconsin/67/05		sw/Gent/172/08[a]		A/Indiana/08/11	
	HI	VN	HI	VN	HI	VN	HI	VN	HI	VN
A/Victoria/3/75	160	960	< 10	< 10	< 10	15	20	120	10	< 10
A/Nanchang/933/95	< 10	20	320	1280	< 10	15	< 10	10	< 10	80
A/Wisconsin/67/05	< 10	10	< 10	20	160	160	< 10	10	< 10	< 10
sw/Gent/172/08	80	80	< 10	< 10	< 10	15	2560	5120	< 10	< 10
A/Indiana/08/11	20	30	< 10	240	< 10	15	10	40	320	2560

HI: hemagglutination-inhibition; VN: virus-neutralization.

[a] Serum against sw/Gent/172/08 was obtained by hyper-immunisation of swine; the other sera were post-infection ferret sera.

[b] Ferret serum to A/Wuhan/359/95 was used, because ferret serum to A/Nanchang/933/95 was not available. The two viruses are antigenically identical.

5.2.4.2 Serological status to human seasonal influenza A(H3N2) viruses

HI antibody titers ≥ 10 against the A/Victoria/3/75 virus were detected in 78% of individuals born before 1990 (Figure 2). In contrast, only 10% of those born in the 1990s and none of the children born after 2000 had detectable antibodies. HI titers ≥ 40 were most common in persons born in the 1950s, 1960s and 1970s. A larger proportion of people had detectable HI antibodies and titers ≥ 40 against the A/Nanchang/933/95 virus. Detectable HI antibodies against the latter virus were observed in 85% of individuals born before 2000, but only in 20% of the children born after 2000. More than half of those born in the 1970s, 1980s and 1990s had HI titers ≥ 40. Seroprevalence rates against the A/Wisconsin/67/05 strain had the least variation across different age groups. More than half of the people in each age category had detectable antibodies, except for those born in the 1960s (42%). HI

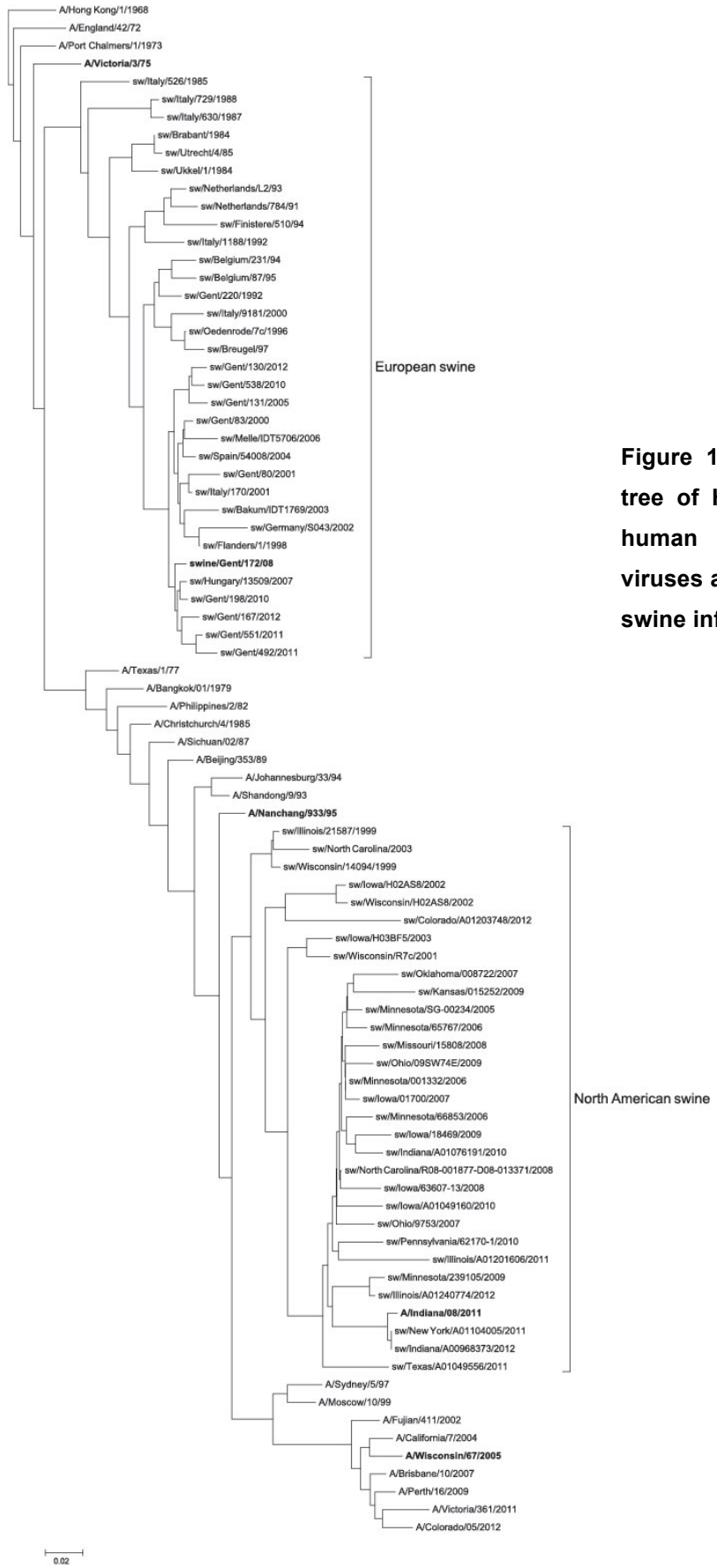
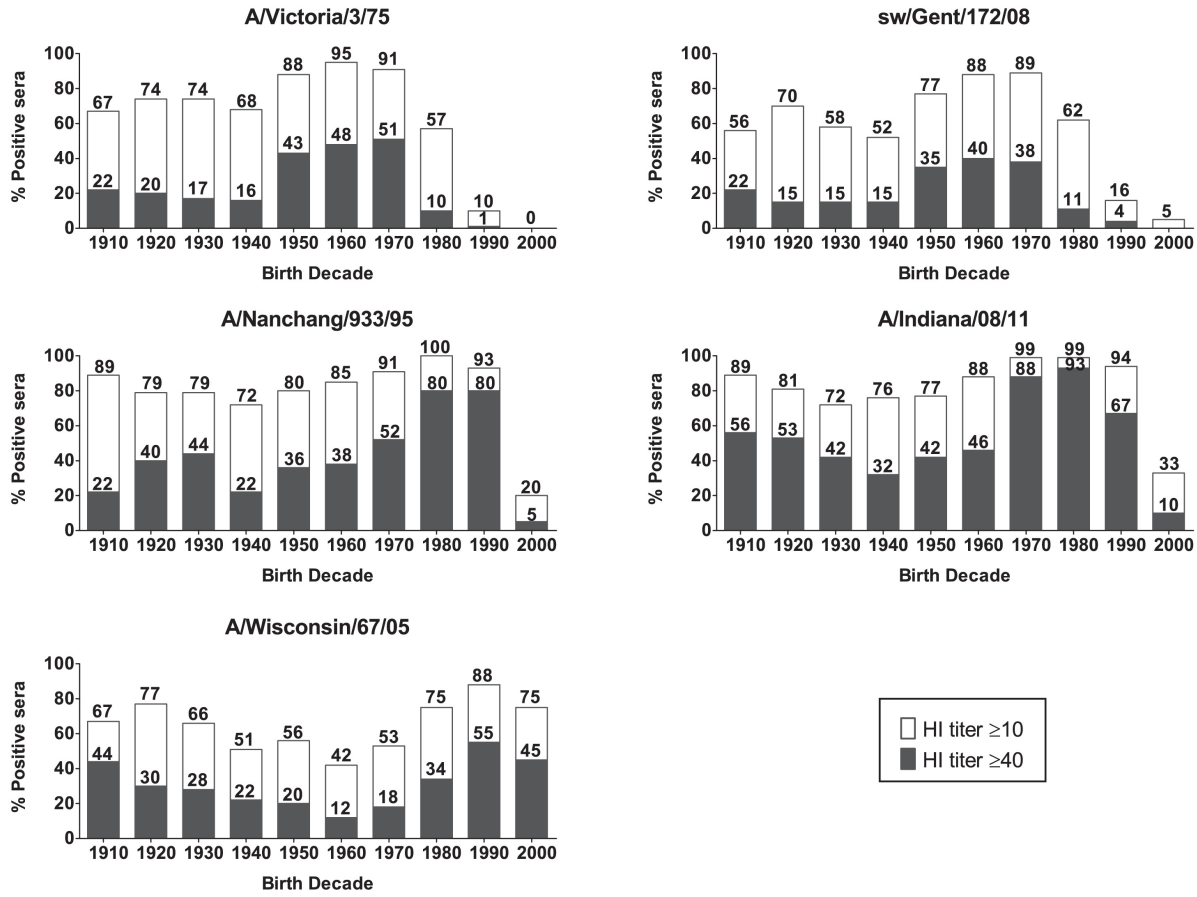


Figure 1. Neighbour-joining phylogenetic tree of HA1 amino acid sequences from human seasonal influenza A(H3N2) viruses and European and North American swine influenza A(H3N2) viruses.

The viruses used for the present serological studies are indicated in bold.



HI: hemagglutination inhibition.
The percentage of individuals with detectable (white bars) and high (black bars) antibody titers are shown. Numbers above the bars represent the percent of positive sera at each cut-off value.

Figure 2. Prevalence of hemagglutination inhibition antibodies against three human seasonal influenza A(H3N2) viruses, an endemic European H3N2 swine influenza virus and a North American H3N2v virus, by decade of birth, Luxembourg, 2010 (n = 843).

titers ≥ 40 were most common in those born after 1990.

GMTs of HI antibodies against the three human seasonal H3N2 viruses showed similar age-dependent trends as the seroprevalence rates (Table 2). Antibody titers against A/Victoria/3/75 were highest in people born in the 1950s, 1960s and 1970s. Antibody titers against A/Nanchang/933/95 and A/Wisconsin/67/05, on the other hand, were highest in people born in the 1980s and 1990s and those born in the 1990s, respectively. Peak antibody titers against A/Nanchang/933/95 were higher than those against either of the other viruses.

Table 2. Haemagglutination inhibition antibody titers against human and swine-origin influenza A(H3N2) viruses, by age group, Luxembourg, 2010 (n = 843)

Birth decade	n	GMT (95% CI)				
		A/Victoria/3/75	A/Nanchang/933/95	A/Wisconsin/67/05	sw/Gent/172/08	A/Indiana/08/11
1910s	9	13.6 (6.4-29.1)	20.0 (7.9-50.3)	25.2 (7.0-90.4)	12.6 (4.3-36.6)	29.4 (10.1-85.6)
1920s	91	13.8 (11.5-16.5)	21.4 (17.1-26.8)	18.0 (14.5-22.3)	12.1 (10.2-14.4)	28.2 (21.8-36.5)
1930s	100	12.7 (10.9-14.8)	22.4 (18.3-27.3)	16.1 (13.0-20.0)	11.2 (9.5-13.2)	22.1 (17.4-28.0)
1940s	100	12.4 (10.5-14.7)	14.9 (12.4-17.8)	12.0 (9.7-14.9)	11.1 (9.2-13.4)	17.6 (14.3-21.6)
1950s	100	25.0 (20.2-30.9)	19.2 (15.6-23.5)	12.8 (10.3-16.0)	16.8 (13.7-20.7)	22.0 (17.3-28.1)
1960s	100	30.1 (24.9-36.5)	24.0 (19.2-29.9)	9.7 (7.9-11.8)	24.1 (19.3-30.1)	28.7 (22.8-36.1)
1970s	100	28.7 (23.5-35.0)	29.1 (23.8-35.6)	10.8 (8.9-13.1)	23.1 (19.1-28.0)	69.6 (58.0-83.7)
1980s	104	10.0 (8.7-11.5)	71.0 (59.1-85.3)	18.2 (14.8-22.4)	10.9 (9.4-12.7)	117.8 (97.5-142.2)
1990s	99	5.5 (5.2-5.8)	77.8 (59.5-101.6)	34.3 (26.9-43.7)	6.2 (5.5-7.0)	58.8 (44.6-77.5)
2000s	40	5.0 (5.0-5.0)	6.5 (5.4-7.8)	20.4 (14.6-28.3)	5.2 (4.9-5.4)	7.7 (6.1-9.7)

GMT: geometric mean titer; CI: confidence interval.

5.2.4.3 Serological status to European and North American swine-origin influenza A(H3N2) viruses

Rates of seroprevalence against the European H3N2 SIV sw/Gent/172/08 and North American H3N2v virus A/Indiana/08/11 were similar to those against the human seasonal viruses A/Victoria/3/75 and A/Nanchang/933/95, respectively (Figure 2). HI antibodies (titers ≥ 10) against sw/Gent/172/08 were almost exclusively found in those born before 1990, of whom 70% were seropositive. HI antibodies against A/Indiana/08/11 were mainly detected in people born before 2000, of whom 86% were seropositive. In contrast, fewer than 20% of those born after 1990 had antibodies against sw/Gent/172/08, and only 33% of children born after 2000 showed antibodies against A/Indiana/08/11. Seroprevalences and HI antibody GMTs followed similar age-specific patterns for both viruses (Figure 2, Table 2). The highest proportions of HI titers ≥ 40 and the highest GMTs were observed in individuals born in the 1950s to 1970s for sw/Gent/172/08, and in those born in the 1970s to 1990s for A/Indiana/08/11. Nonetheless, the prevalence of titers ≥ 40 and the GMTs were higher for A/Indiana/08/11 than for sw/Gent/172/08 in all age categories ($p < 0.05$), except for those born in 1950s and 1960s ($p > 0.05$).

Sera from individuals born after 1940 were also examined for antibodies against the two swine-origin H3N2 viruses with the VN assay (Table 3). For both viruses, VN

titers were higher than HI titers ($p < 0.05$), except for antibodies against sw/Gent/172/08 in children born after 2000 ($p > 0.05$). Higher VN antibody titers were detected against A/Indiana/08/11 than against sw/Gent/172/08 ($p < 0.05$).

Table 3. Virus-neutralizing antibody titers against swine-origin influenza A(H3N2) viruses sw/Gent/172/08 and A/Indiana/08/11 in people born after 1940, Luxembourg, 2010 (n = 643)

		sw/Gent/172/08			A/Indiana/08/11		
		% sera with titer			% sera with titer		
Birth decade	n	≥10	≥40	GMT (95% CI)	≥10	≥40	GMT (95% CI)
1940s	100	77	36	24.0 (18.6-31.0)*	98	71	80.6 (62.6-103.6)*
1950s	100	94	68	76.4 (56.8-102.7)*	96	69	90.1 (67.3-120.6)*
1960s	100	93	62	62.6 (46.6-84.0)*	100	82	112.4 (87.7-144.2)*
1970s	100	99	79	90.4 (70.9-115.1)*	100	94	247.2 (199.7-306.0)*
1980s	104	79	30	22.7 (18.4-28.1)*	100	99	488.1 (410.4-580.5)*
1990s	99	30	9	8.0 (6.7-9.7)*	97	89	235.4 (172.3-321.7)*
2000s	40	10	0	5.4 (5.0-5.7)	58	15	13.3 (9.2-19.2)*

GMT: geometric mean titer; CI: confidence interval.

* GMTs significantly higher ($p<0.05$, by Wilcoxon signed-rank test) in the virus-neutralizing than in the hemagglutination-inhibition assay.

5.2.4.4 Correlations between antibody titers against human seasonal and swine-origin influenza A (H3N2) viruses

There was a strong correlation between HI antibody titers against sw/Gent/172/08 and A/Victoria/3/75 viruses ($r = 0.71$), and between those against A/Indiana/08/11 and A/Nanchang/933/95 viruses ($r = 0.69$) (Table 4). Correlations were low between HI titers against A/Wisconsin/67/05 and sw/Gent/172/08 ($r = 0.25$), as well as between those against A/Wisconsin/67/05 and A/Indiana/08/11 ($r = 0.42$) (all $p < 0.01$).

Table 4. Pearson correlation coefficients between hemagglutination inhibition antibody titers against human and swine-origin influenza A(H3N2) viruses, Luxembourg, 2010 (n = 843)

	A/Victoria/3/75	A/Nanchang/933/95	A/Wisconsin/67/05	sw/Gent/172/08	A/Indiana/08/11
A/Victoria/3/75	1	0.17	0.24	0.71	0.09
A/Nanchang/933/95		1	0.48	0.26	0.69
A/Wisconsin/67/05			1	0.25	0.42
sw/Gent/172/08				1	0.31
A/Indiana/08/11					1

All $p<0.01$.

5.2.5 Discussion

The present study was designed to investigate the extent to which prior exposure, through infection or vaccination, to earlier antigenic variants of seasonal influenza A(H3N2) viruses was associated with the presence of antibodies against swine-origin H3N2 viruses from Europe and North America in people in Luxembourg born between 1910 and 2010. Our results demonstrate that as many as 70% of people in the study born before 1990 had detectable HI antibodies against the European H3N2 SIV, whereas such antibodies were generally lacking in those born after 1990. The prevalence of antibodies against the antigenically distinct H3N2v swine-origin virus was also age-dependent: antibodies were predominantly found in people born before 2000, of whom 86% were seropositive. Our data are consistent with previous studies (CDC, 2012; De Marco *et al.*, 2013; Hoschler *et al.*, 2013; Krumbholz *et al.*, 2014; Skowronski *et al.*, 2012a; Skowronski *et al.*, 2012b; Waalen *et al.*, 2012), but we have for the first time demonstrated a lower level of seroreactivity to the European H3N2 SIV than to the H3N2v virus. Although HI titers of ≥ 40 are generally considered as seroprotective, people with lower antibody titers may also have some protection against H3N2 viruses from swine. Indeed, HI assays do not measure mucosal antibodies, antibodies against NA and cell-mediated immunity, which will also contribute to protection (Tamura *et al.*, 2005). The VN assay yielded higher seroprevalence rates and antibody titers against both swine-origin influenza viruses than the HI assay. This is not surprising, because the VN assay detects a broader range of antibodies than the HI assay (Sui *et al.*, 2009).

We have several reasons to believe that antibodies against current swine-origin H3N2 viruses result from exposure to historical human H3N2 strains rather than from infection with SIVs. Firstly, Luxembourg has a low pig density compared with other European regions and only 0.07% of the population are employed in the swine industry. Furthermore, only 0.99% of the Luxembourg swine population tested seropositive against H3N2 SIV in 2013 (Lutteke *et al.*, 2014). Secondly, H3N2 SIVs from North America have never been detected in swine in Europe. Finally, we found a strong correlation between antibody titers against swine-origin viruses sw/Gent/172/08 and A/Indiana/08/11 and their respective human ancestor A/Victoria/3/75 and A/Nanchang/933/95. Similar correlations have previously been

reported between antibody titers against the European H3N2 SIV and A/Port Chalmers/1/73 (Krumbholz *et al.*, 2014), and between the H3N2v virus and A/Wuhan/359/95 and A/Sydney/5/97 viruses (Hoschler *et al.*, 2013; Skowronski *et al.*, 2012b). In contrast, younger people who had not been exposed to these human H3N2 strains in past decades, were generally seronegative against the swine-origin viruses. In addition, our phylogenetic and antigenic analyses, in agreement with previous studies (Lina *et al.*, 2011; Qiu *et al.*, 2013), confirm the close relationship between these swine-origin viruses and their human H3N2 ancestors.

Despite lower seroreactivity to the European H3N2 SIV than to the North American H3N2v virus, only three human infections with the European H3N2 SIV have been reported between 1993 and 2014 (Claas *et al.*, 1994; Gregory *et al.*, 2001). The H3N2v viruses, in contrast, have caused 343 human infections in the US between 2011 and 2014. It is possible that the H3N2v virus is more infectious for humans than other H3N2 lineages from swine or that more people in the US may have opportunities for exposure to pigs. The H3N2v is the only North American H3N2 SIV genotype that has caused widespread infections in humans, and although some believe this is due to the presence of the pandemic M gene segment, this has not been firmly proven (Van Reeth, 2013). Most H3N2v cases reported exposure to pigs at agricultural fairs (Jhung *et al.*, 2013; Wong *et al.*, 2012). Thousands of fairs are held in summer and autumn in North America, and these fairs provide unique settings where pigs from numerous sources can come into contact with millions of persons, which may facilitate interspecies transmission of influenza viruses. Such large-scale pig shows are rare in Europe. Furthermore, human cases of animal influenza have been notifiable in the US since 2007, which is not the case in Europe, and there is much more extensive surveillance for influenza in humans and swine in the US than in Europe.

Our data further support the notion that pigs serve as reservoirs for older human H3 HAs against which immunity in the human population will gradually decrease over time. Experimental infection studies in pigs and in ferrets have shown that prior infection with recent seasonal H3N2 viruses offers limited or no protection against challenge with the European H3N2 SIV or H3N2v (Houser *et al.*, 2013; Qiu *et al.*, 2013). As such, H3N2 SIVs, and the European strains in particular, could potentially contribute to pandemic viruses in the future as seroreactivity to the respective HAs wanes over time in the human population. Yet, it is highly likely that the current

substantial immunity in people born before 1990 would prevent extensive spread of H3N2 SIVs. As for the swine-origin pH1N1 virus, pre-pandemic antibodies against pH1N1 were present in most individuals born before 1944, but were low or absent in younger people (CDC, 2009; Hancock *et al.*, 2009; Ikonen *et al.*, 2010b; Skowronski *et al.*, 2011). It may take nearly 50 years before a substantial proportion of the human population would be fully susceptible to swine-origin H3N2 viruses. To assure the best preparation for swine-origin influenza virus pandemics, surveillance of influenza in pigs should be expanded and integrated with human public health surveillance efforts, and additional studies on the cross-reactivity between human and swine influenza viruses are warranted.

5.2.6 Acknowledgements

We thank Dr. John McCauley for providing the seasonal H3N2 influenza viruses and H3N2v virus, Dr. Guus Rimmelzwaan for providing the ferret sera, Dr. Christopher Olsen for proofreading our manuscript, and Lieve Sys, Nele Dennequin, Melanie Bauwens and Karl De Hert for technical assistance. This study was supported by the FLUCROSS project (RT 09/6227) of Belgian Federal Service for Public Health, Food Chain Safety and Environment and EU FLUPIG project (FP7-GA 258084).

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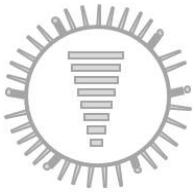

Chapter 6

General Discussion

Heterovariant and heterosubtypic protection between influenza viruses

Our research shows that cross-protection between influenza viruses correlates with the genetic similarities of the viral HA and NA genes. That is, protection is usually stronger against challenge viruses with HA and/or NA proteins of the same origin as the viruses used for primary infection. This is seen in the complete cross-protection between pH1N1 and the European SIV H1avN1 as well as H1huN1, reflecting the same N1 origin of these viruses. Likewise, full cross-protection also occurred between the historic human seasonal H3N2 virus (A/Victoria/3/75) and the European H3N2 SIV, which share both the origin of their H3 and N2. This strong protection can be induced independently by either HA or NA, and it can explain why there may be stronger cross-protection between viruses with a different HA subtype but a closely related NA (heterosubtypic protection, i.e. between H3N2 and H1N2) than between viruses of the same HA and NA subtype (heterovariant protection, i.e. between different H3N2 viruses). As an illustration, after challenge with a European H3N2 SIV, nasal virus shedding and virus replication in respiratory tissues were almost undetectable in pigs previously infected with a European H1huN2 SIV, but they were only minimally reduced in pigs previously infected with a contemporary human seasonal H3N2 virus A/Wisconsin/67/05.

Our results show substantial heterovariant-protection can even occur between two viruses having a significant number of amino acid differences in the HA antigenic sites. For example, 17 and 9 amino acid differences in atnigenic sties were observed between pH1N1 and European H1avN1 SIV, and between A/Victoria/3/75 and European H3N2, respectively. Still, a complete cross-protection occurred in both cases. While the related NA of these viruses has likely contributed to the protection, a closer look at the HA1 genetic sequences revealed that the immunodominant antigenic sites (site A and B in H3, and site Sa in H1) were largely conserved in both cases. We found minimal cross-reactive virus-neutralizing (VN) antibodies to both challenge viruses in serum, but we hypothesize that VN antibodies in the respiratory mucosa may be of high affinity and thereby contribute to a rapid and efficient control of virus replication. This critical protective role of immunodominant antigenic sites has been demonstrated at the time of the 2009 pandemic in humans. The 2009 pH1N1 differs from the 1918 Spanish H1N1 pandemic virus by 10 amino acids in all 4 antigenic sites, but the Sa antigenic site is extremely conserved (only 1 amino acid difference) (Xu *et al.*, 2010). This correlates well with the observation that humans

who have experienced the 1918 Spanish flu carry the highest titers of VN antibodies against the 2009 pH1N1 viruses and have lowest infection rates amongst all age groups (CDC, 2009; Hancock *et al.*, 2009; Skowronski *et al.*, 2011).

In 1990, Wilson and Cox proposed that a drift variant of human seasonal influenza viruses with ≥4 amino acid changes at ≥2 of the 5 antigenic sites would be of epidemiologic importance (Wilson & Cox, 1990). According to more recent studies with H3N2 viruses of humans and of North American swine, 6 or 7 amino acid positions in the HA1 are critical for the viral antigencity. These amino acid positions are identical in both species and 1 or 2 changes of them are believed to result in significant antigenic drift, as determined by cross-HI assays with post-infection ferret sera or hyperimmune swine sera (Koel *et al.*, 2013; Lewis *et al.*, 2014). It is unknown whether those key amino acid positions are also applicable to other H3N2 lineages such as European H3N2 SIVs, and to what extent they really matter in cross-protection in the field. The A/Victoria/3/75 and European H3N2 SIV used in our cross-protection study in pigs have 4 residue differences in those identified positions, and cross-reactivity between them was undetectable in the HI assay, and only minimal in the VN assay. Still, a complete protection against the European H3N2 SIV occurred in pigs previously infected with A/Victoria/3/75. Several studies have shown that 1-3 amino acid changes in the HA1 molecule of H1N1 and H3N2 viruses could possibly reduce the cross reactivity and the efficacy of inactivated vaccine in an animal model (Katz & Webster, 1989; Kodihalli *et al.*, 1995; Newman *et al.*, 1993; Wood *et al.*, 1989). This disagrees with the “high” threshold observed in our experimental pig infection studies. One possible explanation is that experimental inoculation with a high dose of influenza viruses can provoke a broader immune response, i.e. local antibody responses in the respiratory tract, than whole inactivated virus vaccines (Van Reeth & Ma, 2013). However, we have also performed vaccination-challenge experiments with a commercial vaccine based on A/Port Chalmers/1/73 vaccine and challenge with a European H3N2 SIV. The two viruses had 11 amino acid differences in all antigenic sites of which 6 occurred in site A and B. Still, challenge virus titers in the lungs, trachea and nasal mucosa of the vaccinated pigs were significantly reduced after challenge (De Vleeschauwer *et al.*, 2015). We believe that more differences in critical amino acid positions are required for a loss of cross-protection in infection-immune pigs or pigs vaccinated with some

commercial vaccines. Further studies into the most important amino acids for various strains are required.

Traditionally, HA and NA mediated immunity is examined by HI, VN and neuraminidase-inhibition (NI) assays on serum. However, the respiratory tract is the site where influenza virus infection occurs, and is the front line where host immunity clear viruses. Our findings are in accordance with previous reports that the porcine nasal mucosa is the predominant location of both IgG and IgA producing cells after infection with a North American H1N1 SIV (Larsen *et al.*, 2000). In addition, we have shown that protection between two genetically and antigenically distinct swine-origin H3N2 viruses was associated with a rapid boost of antibody-secreting cell responses in the nasal mucosa after challenge. Likewise, human nasal-associated lymphoid tissue is considered the important induction site for both mucosal and systemic immunity to upper respiratory pathogens, including influenza virus (Guthrie *et al.*, 2004; Wiley *et al.*, 2001; Zuercher *et al.*, 2002). All these data spur more studies focusing on the immune responses at the respiratory mucosae. The markedly similar pathogenesis and immune responses to influenza viruses in the respiratory tract in pigs and in humans make the pig an interesting animal model for humans to study this issue.

The ELISpot assays developed in our study were shown to be a valuable tool to quantify local and systematic influenza virus specific immune responses. We have performed basic assays using whole virus preparations to detect the responses of immune cells. The use of split proteins, or synthesized peptides in future studies will allow to get insights into the major viral targets of the immune responses.

In our research, we used the a miniaturization of the thiobarbituric acid (TBA) method to quantify NI antibodies (Sandbulte *et al.*, 2009). We used natural, whole influenza virus as antigen and fetuin, a highly glycosylated protein, as substrate to measure the amount of free sialic acid, the soluble product of NA activity. Other researchers frequently use reassortant viruses that are produced by reverse genetics to contain the relevant NA gene but a novel subtype of HA. This is because sometimes HA-specific antibodies in polyclonal serum may block access of substrate to the NA catalytic site, leading to an overestimated NI titer (Gerentes *et al.*, 1999). However, this unspecific interference role of HA-specific antibodies has not been observed so far in all our studies with post-infection swine sera. As an example, we did not observe any cross-reactivity in the NI assay between H1huN1 and H1huN2 SIVs, in

spite of the two viruses having very similar HAs and cross-reactivity in the HI assay. Considering that cross-reactive HI antibodies against the challenge virus were absent in all our cross-protection studies in this thesis, we believe that the detected cross-reactive NI antibodies were specific to the NA of the challenge virus, and that the association with the observed cross-protection was valid.

Our experimental cross-protection data may explain recent changes in the epidemiology of SIVs in Europe. For instance, pH1N1 is found to be the dominant H1N1 virus in the United Kingdom, where there is a low prevalence of H1avN1. The other way round, pH1N1 is hardly detected in Belgium and the Netherlands where H1avN1 is very widespread (Simon *et al.*, 2014). Furthermore, H3N2 is rare in pigs in Brittany, France, whereas the prevalence of H1huN2 is high in this region (Simon *et al.*, 2014). But there are also some exceptions: Denmark has a similarly high prevalence of H1avN1 and pH1N1, while Germany and Italy have similarly high prevalences of H3N2 and H1huN2.

The geographic diversity of endemic SIV strains, and the dramatic increase in the numbers of novel SIV lineages highlight the need for more broadly protective or universal vaccines. In this respect, live attenuated influenza vaccines (LAIVs) are considered to have some advantages over whole inactivated influenza vaccines (WIVs), which include the stimulation of mucosal antibodies, a cytotoxic T cell response, and longer-lasting immunity (Ambrose *et al.*, 2012; Boyce *et al.*, 1999; Cox *et al.*, 2004). However, studies show that LAIV does not always provide superior protection from heterologous infection compared to WIV. In comparative studies with LAIV and WIV based on a North American cluster I H3N2 SIV, the WIV frequently show a similar reduction in nasal virus shedding and viral titers in lung tissues and bronchoalveolar lavage fluid after challenge with a H3N2 SIV of cluster II or IV (Kitikoon *et al.*, 2013; Loving *et al.*, 2013; Vincent *et al.*, 2012). After intranasal administration of LAIV, researchers have detected mucosal antibodies in nasal mucosa and bronchoalveolar lavage fluid, but the cell-mediated immune responses were inferior in magnitude compared to those observed after infection with wild type virus (Kappes *et al.*, 2012). This can be explained by the minimal replication of the LAIV in the respiratory tract as compared to the wild type virus. Also, in the field, vaccines are usually administered in the context of pre-existing infection immunity, and this raises the question as to how well the LAIV could replicate and induce immunity in an infection-immune host. Our studies indicate that prior infection

immunity impairs the replication of a second virus in spite of inoculation with a dose up to 10^7 50% infection dose (ID_{50}). It is therefore doubtful whether the LAIV, which is usually administrated at a dose of 10^3 ID_{50} will be able to replicate sufficiently to induce a robust immunity against influenza.

Zoonotic potential of European SIVs

All three influenza pandemics of the 20th century were initiated by antigenic shift: the introduction of a new HA subtype from an avian reservoir in the human population. The 2009 H1N1 pandemic was different because for the first time a distinct variant of a currently circulating HA subtype in humans triggered a new pandemic (Garten *et al.*, 2009; Itoh *et al.*, 2009). It also highlights the importance of swine as reservoir for pandemic viruses.

Our study in pigs has demonstrated that prior infection with pH1N1 can induce robust protection against European H1avN1, H1huN1, and H1huN2 SIVs, but had little impact on the European H3N2 SIV. It is likely that the current high population immunity against pH1N1 in humans will enhance their cross-protective immunity to European H1 SIVs, making those viruses less likely to cause pandemics in the near future. The public health importance of H3N2 SIVs has been highlighted after >350 human infections with H3N2v during 2011-14 in the United States (US). Similar to pH1N1 virus, most if not all H3N2 SIVs have their HA derived from human seasonal H3N2 viruses of different years. Due to the much slower antigenic drift rate of H3N2 viruses in swine than in humans, seasonal human H3 HAs gradually diverged from the swine lineage (de Jong *et al.*, 2007; Lewis *et al.*, 2014). As a result, the young population has only been exposed to the contemporary seasonal viruses and they may lack protective immunity against H3N2 SIVs. This hypothesis is supported by our study showing a complete virological protection against infection with a European H3N2 SIV in pigs infected with an old human seasonal H3N2 virus (A/Victoria/3/75), but only a minimal protection in pigs pre-infected with the more recent human seasonal H3N2 virus (A/Wisconsin/67/05). Our serological study further shows that the prevalence of HI antibodies against the European H3N2 SIV and H3N2v were age-dependent, and strongly correlated with the prevalence of HI antibodies against their ancestor human viruses A/Victoria/3/75 and A/Nanchang/933/95, respectively. Individuals born after 1990 and 2000 generally lacked detectable HI antibodies (titer $\geq$10) against the European H3N2 SIV and H3N2v, respectively.

Our findings fit with previous H3N2v serological studies (Hoschler *et al.*, 2013; Skowronski *et al.*, 2012a; Skowronski *et al.*, 2012b), and with the epidemiological fact that H3N2v mainly infected children born after 2000 (Epperson *et al.*, 2013; Jhung *et al.*, 2013). Though the prevalence of antibodies against the European H3N2 SIV was lower compared to that against H3N2v, only three infant infections with the former virus have been documented since 1993 (Claas *et al.*, 1994; Gregory *et al.*, 2001). But due to the lack of routine surveillance in people in contact with swine in Europe, the real zoonotic infection rates are likely to be higher. Still, Europe does not have agricultural fairs with swine exhibition as in the US, which has proven to be an important site for the interspecies infections of influenza viruses across the US (Bowman *et al.*, 2014; CDC, 2012; Killian *et al.*, 2013; Wong *et al.*, 2012). The history of influenza pandemics and epidemics, as well as the emergence of H3N2v, repeatedly demonstrate viral evolution through genetic reassortment may aid viral invasion and persistence in the new host. Thus, continued surveillance of virus epidemiology and human population immunity is required to ensure a timely response in case of H3N2 SIV outbreaks in humans.

The zoonotic potential of H3N2 SIVs of European and North American lineages has been discussed by our and others' studies, but H3 viruses from other animal reservoirs should not be ignored. Identifying such strains is of paramount value for pandemic surveillance and preparedness. Similar to H3N2 SIVs, the HA genes of the H3 subtype isolated from ducks are conserved antigenically and genetically with a markedly lower rate of silent and coding nucleotide substitution than for human H3 strains, with amino acid substitutions occurring outside recognized antibody binding sites (Kida *et al.*, 1987). Also, sequencing studies of equine viruses of the H3 subtype have shown that the HA of these viruses evolved genetically in a pattern very similar to that for human H3N2 viruses (Kawaoka *et al.*, 1989; Murcia *et al.*, 2011), albeit at a slower rate of antigenic drift (Lewis *et al.*, 2011). The antigenic gap between those animal H3 viruses and human seasonal viruses may pose a threat to public health. Similar cross-protection and serosurveillance studies in both species as for H3N2 SIVs are required to evaluate their potential zoonotic risks.

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Summary-Samenvatting

Summary

Swine influenza viruses (SIVs) of H1avN1, H1huN2, and H3N2 lineages, as well as the more recently established 2009 pandemic H1N1 (pH1N1) lineage are endemic in the European swine population. The H1huN2 and H3N2 SIVs have HAs and NAs from historical human seasonal viruses that have already disappeared from humans for decades. The pH1N1 is circulating in humans and swine worldwide, whereas all other three lineages have a different genetic make-up and antigenic properties from the influenza A viruses currently circulating in humans as well as in pigs of other continents. Therefore, SIVs from other continents may pose a substantial threat to the European swine population. Similarly, European SIVs may also pose a threat for introduction/re-introduction into the human population, with associated outbreaks and pandemic implications.

In **chapter 1**, a general introduction to influenza A virus classification, virion structure and viral evolution is given. The second section presents the epidemiology of SIVs in Europe and North America, with an emphasis on the H3N2 SIVs. The antigenic differences between human and swine H3N2 viruses, and human infections with H3N2 SIVs are also reviewed. The third section presents a summary of the current knowledge of the pathogenesis and immune responses to influenza viruses, especially cross-protection between antigenically distinct viruses. In the fourth section, pros and cons of the pig model for influenza research are summarized and compared with other laboratory animal models.

Chapter 2 presents the aims of the thesis. The main aim was to contribute to the general understanding of cross-protective immunity between influenza viruses, and to evaluate the zoonotic potential of European SIVs.

Chapter 3 characterizes the genetic and antigenic evolution of European H3N2 SIVs from 1998-2012. The European H3N2 SIVs had a similar nucleotide substitution rate as compared to their human and North American swine counterparts in both HA and NA segments. But in contrast to their human and North American swine counterparts, European SIVs showed little changes in antigenic sites and antigenic properties of HA and NA. The results are presented as a report because the study still needs to be completed.

Chapter 4 deals with cross-protection between SIVs of the same HA and NA subtypes (heterovariant protection), or between SIVs of different HA and/or NA subtypes (heterosubtypic protection). In **chapter 4.1.**, we assessed whether pigs after infection with a European H3N2 SIVs would be protected against infection with an antigenically distinct North American H3N2 variant virus (H3N2v). The latter virus has so far never been isolated in Europe, but it may be introduced by export of pigs from the United States (US) to other continents. The H3N2v, is a swine-origin virus and it has all but the matrix gene segments of a phylogenetically distinct origin from the European H3N2 SIV. Influenza naïve pigs were first inoculated intranasally with a European H3N2 SIV and 8 weeks later challenged with a H3N2v virus. Infection with the European H3N2 SIV induced minimal H3N2v virus-neutralization (VN) antibodies, and no hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) antibodies in serum. Cross-reactive IgA and IgG antibody secreting cells (ASCs) and IFN-γ secreting cells (SCs) specific to H3N2v were also demonstrated, especially in nasal mucosa. Post H3N2v challenge, the magnitude and duration of virus excretion and virus titers in respiratory tissues at day 3 post-challenge were significantly reduced in European H3N2 SIV-inoculated pigs. This was associated with vigorous early IgG and IgA ASC boost responses to H3N2v, which were by far most pronounced in the nasal mucosa. Our data shows a partial protection between H3N2 viruses of two distinct lineages, and supports the notion that pig is a valuable model to study mucosal immune responses and to improve our understanding of broad-spectrum immunity to influenza.

In **chapter 4.2.**, we examined the extent of virological protection between a) the pH1N1 virus and three different European H1 lineages (H1avN1, H1huN1, and H1huN2), and b) these H1 viruses and a European H3N2 SIV. The increasing number of novel influenza subtypes and genetic variants isolated from pigs has spurred an interest into the extent of cross-protective immunity between them. Also, such study may give us some indications as to whether the high human population immunity to pH1N1 may enhance cross-protection against other European H1 SIVs. The NA of pH1N1 is of European N1 lineage, and the classical HA of pH1N1 has shown to be more closely related to the avian-like H1 than to the human-like H1. Pigs were inoculated intranasally with representative strains of each virus lineage with 6- and 17-week intervals between H1 inoculations and between H1 and H3 inoculations,

respectively. Prior infection of pH1N1 conferred complete protection against H1avN1, a nearly full protection against H1huN1, and a partial but still substantial protection against H1huN2. Protection against H3N2 was weak in pH1N1- or H1N1-immune pigs, but much more solid in those with immunity to H1N2, reflecting the same N2 origin of those two viruses.

Our data suggest post-infection immunity may offer substantial cross- protection against viruses of the same HA and/or NA subtype. Heterosubtypic protection between viruses with both HA and NA of different subtypes, in contrast, appears to be minimal in pigs. Based on our data, we assumed that, the global spread of pH1N1 in humans may also induce enhanced immunity to European H1 SIVs, but have limited impact on the European H3N2 SIV.

In **chapter 5**, the zoonotic potential of European H3N2 SIVs was assessed. In **chapter 5.1.**, the extent of cross-protection between human and swine H3N2 viruses was investigated. H3N2 influenza viruses circulating in humans and European pigs originate from the pandemic A/Hong Kong/68 virus. Because of slower antigenic drift in swine, the antigenic divergence between swine and human viruses has been increasing. It remains unknown to what extent this results in a reduced cross-protection between recent human and swine H3N2 influenza viruses. Using the pig model of influenza, we examined the effect of prior infection with an old (1975) or more recent (2005) human H3N2 virus on protection against challenge with a contemporary European H3N2 SIV. Prior infection with the old human virus offered complete virological protection against challenge. Pigs previously infected with the recent human virus had similar virus titers in the respiratory tract as the previously uninfected challenge controls, but the mean duration of nasal virus excretion was shortened by 1.2 day. This rapid viral clearance was associated with a more rapid development of VN antibodies to the challenge virus than in challenge controls. Our study suggests that European H3N2 SIVs may pose a threat to young people who have only been exposed to the recent human seasonal H3N2 viruses. The older population may have some residual immunity resulting from previous exposure to historical seasonal H3N2 viruses that are related to European H3N2 SIVs.

In **chapter 5.2.** we studied the prevalence of antibodies against H3N2 SIVs in the human population. This study served to further confirm the age-dependent immunity

to H3N2 SIVs in humans. Serum samples, which were collected from humans (n=843) aged 0 to 100 years in 2010 in Luxembourg, were analyzed by HI and VN assays against a European H3N2 SIV, a North American H3N2v virus, and human seasonal H3N2 viruses isolated in 1975, 1995 and 2005. HI antibodies (titer ≥10) to the European H3N2 SIV were almost exclusively detected in those born before 1990, of which 70% were seropositive. HI antibodies to H3N2v were predominantly found in those born before 2000, of which 86% were seropositive. The same pattern was reflected in the VN assay. A strong correlation between HI titers to the European H3N2 SIV and H3N2v and their respective human ancestors, A/Victoria/3/75 and A/Nanchang/933/95 was observed. This finding and the minimal contacts between humans and pigs in Luxembourg suggests that the anti-SIV antibodies detected in the human sera are due to serologic cross-reactivity with historical human H3N2 viruses. The higher prevalence of antibodies to H3N2v than to the European H3N2 SIV suggests that a larger proportion of human population is immunologically susceptible to the latter virus, which would be potentially more damaging than H3N2v if it would jump to humans and acquire the capacity to spread between humans.

In **chapter 6**, all experimental studies are discussed. In general, our study indicates cross-protection between influenza viruses in pigs correlates well with viral genetic and antigenic differences. Although we found mucosal immune B cell responses were associated with broad protection, it remains questionable which immune components best correlate with protection. Prior infection with pH1N1 can generate cross-protective immunity against European H1N1 and H1N2 SIVs, making them less likely to be the candidates for the next pandemic. In comparison, susceptibility to European H3N2 SIVs exists in humans born after 1990, who have never been exposed to H3N2 SIVs or antigenically related viruses. The recent outbreaks of the swine-origin H3N2v in young people in the US also highlights the zoonotic potential of European H3N2 SIVs. Further studies are required to identify the conserved epitopes between human and swine H3N2 viruses, as well as the strategies to enhance human immunity against H3N2 SIVs.

Samenvatting

Varkensinfluenzavirussen (swine influenza viruses, SIV) van de H1avN1, H1huN2 en H3N2 lijnen én de recent ontstane 2009 pandemische H1N1 (pH1N1) lijn komen endemisch voor in de Europese varkenspopulatie. De H1huN2 en H3N2 SIV's beschikken nog steeds over de HA's en NA's van historische humane seizoensgebonden virussen die bij de mens sedert enkele decennia niet meer teruggevonden worden. De pH1N1 circuleert zowel bij mens en varken over de hele wereld, terwijl de andere drie types, die momenteel circuleren op andere continenten, andere genetische en antigene eigenschappen van het influenza A-virus vertonen tussen mens en varken. Daarom kunnen SIV's van andere continenten een gevaar voor de Europese varkenspopulatie vormen. Bovendien kunnen de Europese SIV's ook een bedreiging vormen voor de (her)introductie in de humane populatie, met bijbehorende uitbraken en pandemische implicaties.

Hoofdstuk 1 is een algemene inleiding met weergave van de klassificatie, de virion structuur en evolutie over influenza A virussen. In het tweede deel wordt er uitgebreid over de epidemiologie van SIV's en voornamelijk H3N2 SIV. De antigene verschillen tussen humane en porciene H3N2 virussen, en humane infecties met H3N2 SIV komen alsook aan bod. Het derde deel vat de huidige kennis over de pathogenese en de immuunreacties tegenover influenzavirussen samen, waarbij de nadruk gelegd wordt op de kruisbescherming tussen antigenisch verschillende virussen. In het vierde deel worden de voor-en nadelen van het varken als model voor influenza-onderzoek opgesomd en wordt het varken vergeleken met andere proefdieren.

In **hoofdstuk 2** worden de doelstellingen van de thesis uiteengezet. Het bijdragen aan de algemene kennis over kruisbeschermende immuniteit van influenzavirussen en de evaluatie van het zoönotisch karakter van Europese SIV's waren de hoofddoelstellingen van dit proefschrift.

Hoofdstuk 3 wordt gekenmerkt door de genetische en antigene evolutie van de Europese H3N2 SIV's van 1998-2012. De Europese H3N2 SIV's hadden een vergelijkbare nucleotide substitutiegraad met humane en Noord-Amerikaanse varkenstegenhangers in zowel HA als NA segmenten. In tegenstelling tot humane en Noord-Amerikaanse porciene tegenhangers, toonden Europese SIV's weinig

veranderingen in antigene plaatsen en eigenschappen van HA en NA. De resultaten worden weergegeven als een verslag, aangezien de studie nog afgerond moet worden.

De kruisbescherming tussen SIV's van dezelfde HA en NA subtypes ("heterovariant bescherming") of tussen SIV's met verschillende HA en/of NA subtypes ("heterosubtypische bescherming") wordt beschreven in **hoofdstuk 4**. In **hoofdstuk 4.1.** wordt de eventuele bescherming van varkens na infectie met een Europees H3N2 SIV tegenover een antigenisch verschillend Noord-Amerikaans H3N2 variant virus (H3N2v) nagegaan. Dit laatst vernoemde virus is tot nu toe nog nooit geïsoleerd in Europa, maar het kan door de export van varkens uit de Verenigde Staten (VS) naar andere continenten worden ingevoerd.

Het H3N2v-virus, afkomstig van varkens, heeft enkel de matrix-gen segmenten gemeenschappelijk met een fylogenetisch verschillende Europese H3N2 SIV. Influenza naïeve varkens werden eerst intranasaal geïnoculeerd met een Europees H3N2 SIV 8 weken voor challenge met een H3N2v virus. Infectie met het Europese H3N2 SIV leidde tot een minimale opbouw van H3N2v virus-neutraliserende (VN) antistoffen, en geen hemagglutinatie-inhibitie (HI) en neuraminidase-inhibitie (NI) antistoffen in serum. Kruisreactieve IgA en IgG-antistof uitscheidende cellen (antibody secreting cells, ASC) en IFN-γ uitscheidende cellen (secreting cells, SC's) specifiek voor H3N2v werden eveneens aangetoond, voornamelijk ter hoogte van het neusslijmvlies. De omvang en duur van virusuitscheiding en virustiters in respiratoire weefsels waren aanzienlijk verminderd in Europees H3N2 SIV-immune dieren drie dagen na H3N2v challenge. Dit ging gepaard met sterke vroege "IgG en IgA ASC recall reacties" op H3N2v, die verreweg het meest uitgesproken waren in de nasale mucosa. Onze gegevens tonen een gedeeltelijke bescherming tussen H3N2 virussen van twee verschillende lijnen, en toont aan dat het varken een waardevol model is om mucosale immuunreacties te bestuderen en onze kennis over breed-spectrum immuniteit tegen influenza te verbeteren. In **hoofdstuk 4.2.** wordt de mate van virologische bescherming tussen a) het pH1N1 virus en drie verschillende Europese H1 lijnen (H1avN1, H1huN1 en H1huN2), en b) deze H1 virussen en het Europees H3N2 SIV bestudeerd. Het toenemend aantal nieuwe influenza subtypes en genetische varianten bij varkens wakkerde de interesse in kruisbeschermende

immuniteit aan. Dergelijke studie geeft bovendien een indicatie over of de hoge humane populatieimmuniteit tegenover pH1N1 kan bijdragen tot een kruisbescherming tegen andere Europese H1 SIV's. De NA van pH1N1 is van Europese afkomst en de klassieke HA pH1N1 blijkt nauwer verwant te zijn met de aviaire H1 dan met de humane H1. De varkens werden intranasaal geïnoculeerd met representatieve stammen van elke lijn met een interval van 6 en 17 weken tussen H1 inoculaties respectievelijk tussen H1 en H3 inoculaties. Voorafgaande infectie met pH1N1 resulteerde in volledige bescherming tegen H1avN1, een bijna volledige bescherming tegen H1huN1, en een gedeeltelijke, maar nog steeds aanzienlijke bescherming tegen H1huN2. Bescherming tegenover H3N2 was zwak in pH1N1- of H1N1-immune varkens, en matige bescherming werd gevonden in H1N2-immune dieren, als gevolg van dezelfde N2 oorsprong van die twee virussen.

Onze gegevens suggereren dat immuniteit ontstaan na infectie een aanzienlijke bijdrage kan leveren in kruisbescherming tegen virussen van dezelfde HA en/of NA subtype. Heterosubtypische bescherming tussen virussen met zowel HA en NA van verschillende subtypen blijkt daarentegen bij varkens minimaal te zijn. Op basis van onze gegevens, veronderstellen we dat de wereldwijde spreiding van pH1N1 bij de mens zal bijdragen tot een sterkere immuniteit tegen Europese H1 SIV's, maar een beperkte impact op de Europese H3N2 SIV immuniteit zal hebben.

In **hoofdstuk 5** werd het zoönotische karakter van de Europese H3N2 SIV's beoordeeld. De mate van kruisbescherming tussen humane en porciene H3N2 virussen werd onderzocht in **hoofdstuk 5.1.**. H3N2 influenza virussen, circulerend bij mensen en bij Europese varkens, zijn afkomstig van het pandemisch A/Hong Kong/68 virus. Omwille van de tragere antigene drift bij varkens, zijn de antigene verschillen tussen de porciene en de humane virussen toegenomen. Tot op heden is het onbekend hoeverre dit leidt tot een vermindering van kruisbescherming tussen recente humane en porciene H3N2 influenzavirussen. Gebruikmakend van het varkensmodel voor influenza, hebben we het effect van eerdere infectie met een oude (1975) of recente (2005) humane H3N2 virus op een challenge met een hedendaags Europees H3N2 SIV bestudeerd. Voorafgaande infectie met het oude humane virus resulteerde in volledige virologische bescherming tegen challenge. Varkens die eerder geïnfecteerd werden met het recente humane virus vertoonden

dezelfde virustiters in de ademhalingswegen als de eerder niet-geïnfecteerde controle dieren, maar de gemiddelde duur van virusuitscheiding verkortte met 1.2 dagen. Dit werd geassocieerd met een snellere ontwikkeling van VN antistoffen tegen het challengevirus dan in challenge controle dieren. Onze studie suggereert dat de Europese H3N2 SIV's een gevaar kunnen vormen voor jonge mensen die enkel zijn blootgesteld aan de recente humane seizoensgebonden H3N2 virussen. De oudere populatie daarentegen kan nog beschikken over een immuniteit uit eerdere blootstelling aan historische seizoengebonden H3N2 virussen die zijn gerelateerd aan de Europese H3N2 SIV's.

In **hoofdstuk 5.2.** wordt de prevalentie van antistoffen tegen H3N2 SIV in de humane populatie weergegeven. Deze studie bevestigde de leeftijd-afhankelijke immuniteit voor H3N2 SIV's bij de mens. Serumstalen, die werden verzameld bij mensen van 0 tot 100 jaar (n=843) in 2010 in Luxemburg, werden geanalyseerd met behulp van HI en VN testen tegen een Europees H3N2 SIV, een Noord-Amerikaans H3N2v-virus, en humaan seizoensgebonden H3N2 virus geïsoleerd in respectievelijk 1975, 1995 en 2005. HI antistoffen (titer ≥10) tegenover Europese H3N2 SIV's werden bijna uitsluitend gedetecteerd in degenen die vóór 1990 zijn geboren, waarvan 70% seropositief bevonden werd. HI antilichamen tegenover H3N2v werden voornamelijk gevonden in degenen die zijn geboren vóór 2000, waarbij 86% seropositief bleek. Hetzelfde patroon werd teruggevonden in de VN-test. Een sterke correlatie tussen HI titers van het Europees H3N2 SIV en H3N2v en hun humane voorouders, A/Victoria/3/75 en A/Nanchang/933/95 werd waargenomen. Deze bevinding en het minimaal contact tussen mens en varken in Luxemburg suggereert dat de anti-SIV antistoffen in de humane sera het gevolg zijn van serologische kruisreactiviteit met historische humane H3N2 virussen. De hogere prevalentie van antilichamen ten opzichte van H3N2v in vergelijking met de Europese H3N2 SIV bewijzen dat een groot deel van de humane populatie immunologisch gevoelig is voor de virussen die potentieel schadelijker dan H3N2v zijn als deze direct naar en tussen de mens kunnen spreiden.

In **hoofdstuk 6** worden alle experimentele studies besproken. In het algemeen tonen onze studies aan dat de kruisbescherming tussen influenzavirussen in varkens goed correleert met virale genetische en antigene verschillen. Hoewel mucosale immune

B-cel responsen werden geassocieerd met een brede bescherming, blijft het de vraag welke immuunsysteemcomponenten best correleren met bescherming. Voorafgaande infectie met pH1N1 kan kruisbeschermende immuniteit ten opzichte van Europese H1N1 en H1N2 SIV's teweegbrengen, wat maakt dat deze virussen heel onwaarschijnlijk een volgende pandemie zullen veroorzaken. Ter vergelijking, gevoeligheid voor Europese H3N2 SIV is aanwezig bij mensen geboren na 1990, die niet zijn blootgesteld aan H3N2 SIV of gerelateerde virussen. De recente uitbraken van H3N2v, van varkensoorsprong, bij jonge mensen in de VS, wijst ook op het zoönotische potentieel van de Europese H3N2 SIV's. Verdere studies zijn nodig om de bewaarde epitopen tussen humane en porciene H3N2 virussen te identificeren, alsook strategieën te bepalen om immuniteit tegen humaan H3N2 SIV verbeteren.

Curriculum vitae

Yu Qiu was born in Yancheng, China on November 20, 1986. In 2004, she started her veterinary studies at Yangzhou University, China. In 2006, she joined the Laboratory of Veterinary Microbiology as an undergraduate student research assistant. During 2006-07, she performed research to develop an indirect enzyme-linked immunosorbent assay (ELISA) for detecting antibodies against equine coronavirus. This test was later applied in the equine quarantine for the Beijing 2008 Olympic Games. In 2007, she was awarded with the highest rank scholarship for undergraduate students - National Scholarship offered by the Chinese Education Ministry. After having graduated in 2008, she was recommended to the Graduate School of Yangzhou University waived of entrance exams, and she became an MSc student in the Laboratory of Veterinary Microbiology. Her MSc study focused on the epidemiology and development of diagnostic techniques for avian leukosis viruses (ALVs), which was part of China's national poultry ALVs eradication program. She finished her MSc thesis in December 2010. In January 2011, she joined the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University where she started her PhD research. Her research focused on the cross-protection between various swine influenza viruses, as well as between human and swine influenza viruses, with implications for veterinary and public health. She has published several first-author and co-author papers in international peer-reviewed journals, and has made oral or post presentations in various scientific symposiums.

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Acknowledgements

At the end of this thesis, I would like to express my appreciation to people who have generously contributed to the work presented in this thesis.

First and foremost, I want to appreciate my advisor Prof. Dr. Kristien Van Reeth. Thank you for providing me with the opportunity to pursue the PhD research, and for allowing me to grow as an independent researcher. I sincerely appreciate your contributions of time, ideas, and funding to make my PhD research experience productive and stimulating. I would also like to thank my guidance and examine committee members, Prof. Dr. Maurice Pensaert, Prof. Dr. Xavier Saelens, Prof. Dr. Steven Van Gucht, Prof. Dr. Eric Cox, Prof. Dr. Guus Rimmelzwaan and Dr. Annebel De Vleeschauwer for your time, interests, and brilliant comments on my thesis. I am also grateful to Caroline Bonckaert for translating the summary of this thesis into Dutch.

I want to acknowledge all present and past members of the Laboratory of Virology, for your help and support in my PhD study. I really enjoy the working atmosphere in this lab. Special mention goes to Lieve Sys, Melanie Bauwens and Nele Dennequin for your excellent assistance in the lab work. To Zeger Van den Abeele, Loes Geypen, and Bart Ellebaut for your help in animal experiments. And to Dr. Karen van der Meulen and Dr. Gerwin Claes for helpful discussions. I am also very grateful to Dr. Sjouke Van Pouke and Dr. Zhongfang Wang for your good advices. And to Ivan Trus and Dirk Demeulenaere for your excellent help on photography and computer technology.

I would also like to take this opportunity to appreciate my friends: Sjouke, Zhongfang, Pepe, Karl, Jolien, Liping, Amy (Xiaoyun) and Ivan. We encouraged each other during the difficult times of research, and this has become my very fond memories in Ghent. My time in the lab has been largely enriched by your friendship and accompany, and I hope all of you would remain my lifelong friends. Besides, my stay in Belgium has been made enjoyable in a large part due to many Chinese friends. I am grateful for the time spent with you for travelling together and weekend dinners.

I gratefully acknowledge Belgian Federal Service for Public Health, Food Chain Safety and Environment for the financial support that made my PhD work possible.

Lastly, I would like to thank my family for all their unconditional love and continuous encouragement. For my parents and grandparents who raised me with a love of science and support me in all my pursuits. For my parents in law who understand me and support me in all aspects of life. And most of all for my loving, supportive, encouraging, and patient husband Yewei Li, whose faithful support during the last two years of this PhD is sincerely appreciated. Thank you.